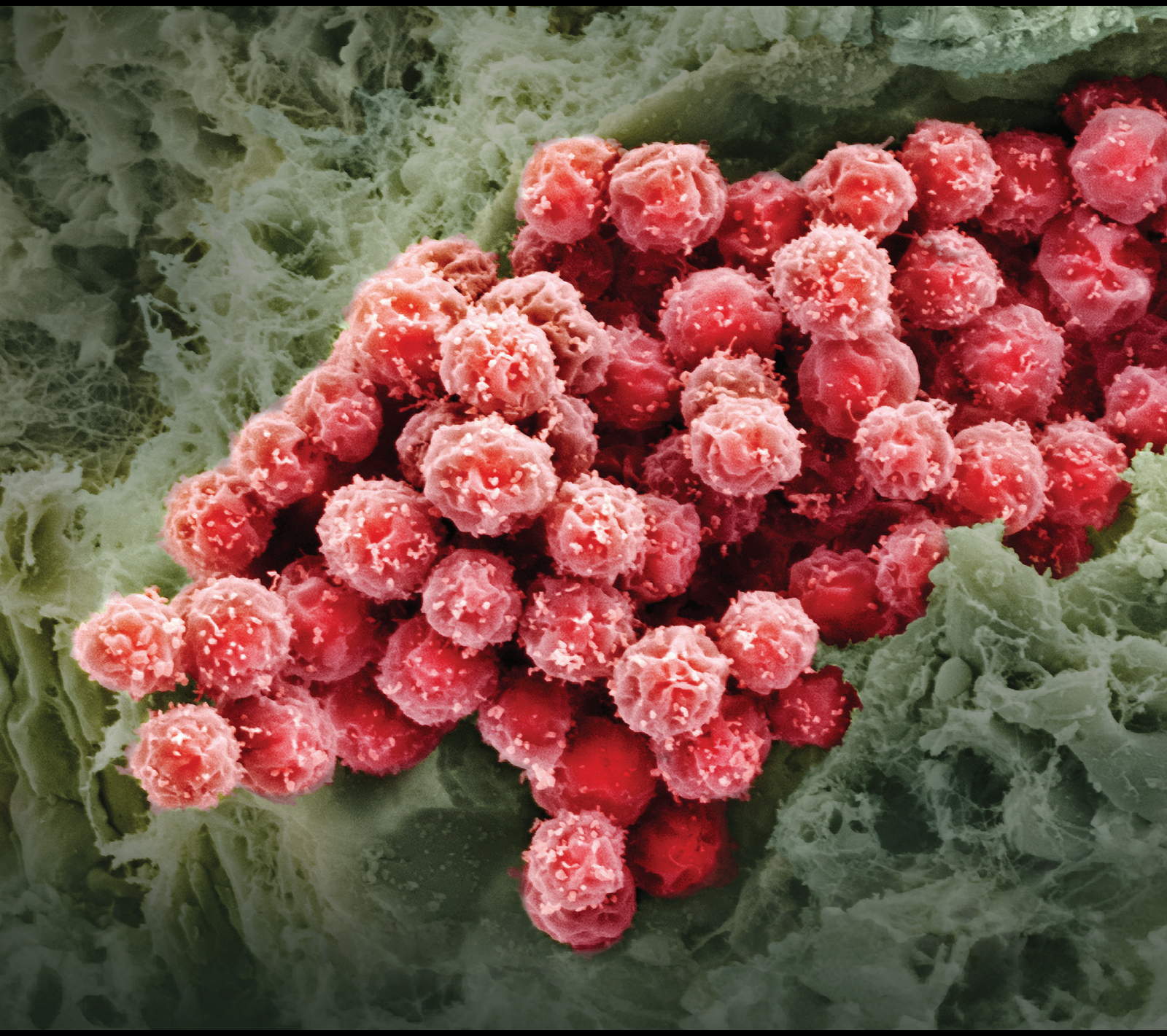
Stem Cells International

Stem Cells in Translational Cancer Research

Guest Editors: Oswaldo Keith Okamoto, Ander Matheu, and Luca Magnani



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Editorial Board

Contents

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Editorial

Stem Cells in Translational Cancer Research

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Since the seminal studies by Fialkow et al. in the seventies [1] and by Bonnet and Dick in the nineties [2], where an organized tumor cell hierarchy originating from transformed hematopoietic stem cells was found in myeloid leukemias, the cancer stem cell (CSC) model of tumor development drew the attention of the scientific community and became the focus of extensive discussion. This theme gained momentum over the past decade, with the identification of stemlike cells as drivers of tumor initiation, recurrence, and metastasis spread in a variety of nonhematopoietic human cancers. These characteristics postulated them as novel and promising therapeutic targets for cancer, especially in the context of drug-resistant tumors [3].

The origin of these stemlike cells is being extensively studied. In some types of tumors, such as in blood, brain, colon, and skin cancer, it has been shown that adult stem cells are prone to transformation due to a longer half-life within tissues compared to their cell progenies, facilitating the accumulation of oncogenic mutations as a result of prolonged exposure to genotoxic stresses [4]. In other cases, *de novo* acquisition of stem cell properties may occur in either normal or neoplastic cells due to genetic/epigenetic aberrations [5]. Finally, the CSC model has been put forward to explain why tumors like breast cancer might return years after surgery. In this situation, lower proliferation rate might allow cells to escape from aggressive therapy and allow for genetic/epigenetic evolution.

Advances in the field, however, point to varied and more complex models of tumor development than previously envisioned, in which the stemlike phenotype may also be dynamically acquired by cancer cells through interaction with stromal cells and soluble factors [6]. A relevant question timely addressed in this special issue is the understanding of the contribution of resident stem cells to the tumor microenvironment (TME). This is of major relevance since they may significantly influence tumor progression, aggressiveness, and response to therapy. The contribution of pericytes to tumor growth, angiogenesis, metastasis, and evasion of immune destruction, which are classic hallmarks of cancer, is comprehensively reviewed by A. L. Ribeiro and O. K. Okamoto. In their article, the authors also discuss a pericyte-mediated regulation of stemness properties in cancer cells and argue in favor of pericytes as cellular targets for new cancer therapies aiming at the TME. Indeed, many new anticancer therapies targeting the TME are under development, with currently approved antiangiogenic drugs as examples of such strategy. However, events of tumor recurrence and poor response to antiangiogenic therapy still puzzle researchers and emphasize the need for continuous studies. In the article by M. Marçola and C. E. Rodrigues, the involvement of endothelial progenitor cells in tumor angiogenesis is critically reviewed. In their article, the specific roles of different members of the VEGF family of growth factors, as well as the relevance of the vascular niche to stemness in cancer cells, are also carefully discussed.

Another practical issue of clinical relevance is the rigorous evaluation of potential oncogenic risks associated with stem cell therapy. Long-term safety issues must be properly addressed before stem cell-based therapies enter clinical trials, but such studies are outnumbered in the literature by

studies evaluating therapeutic effects based on restoration of tissue integrity and physiological balance. While the original article by T. Jazedje et al. addresses this issue at the preclinical stage, showing in a murine breast adenocarcinoma model that mesenchymal stromal cells (MSC) may exert either pro- or antitumorigenic effects depending on the experimental condition, R. Schweizer et al. bring a more clinical perspective to this issue, presenting an interesting discussion about the possible oncogenic hazards of adipose-derived MSC in breast cancer patients subjected to breast reconstruction after mastectomy.

Finally, due to their tumor homing properties, the use of stem cells as vehicles to deliver suicide genes is a strategy highly pursued in cancer gene therapy. However, low delivery efficiency and off-target effects are common limitations of current expression systems. In the original article by Y. Luo et al., the authors elegantly characterize a novel inducible transgene expression system based on the action of neural stem cells that could be further explored to treat malignant gliomas.

In summary, the articles compiled in this special issue highlight how the growing investigation of tumor development through the lens of stem cell biologists is significantly impacting basic and translational cancer research. This interplay between stem cell and tumor biology offers an exceptional opportunity to improve our knowledge about cancer, one of the leading causes of death worldwide. Advances in this field are expected to bring significant impacts in cancer diagnosis and therapy.

Oswaldo Keith Okamoto
Ander Matheu
Luca Magnani

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Research Article

A Double-Switch Cell Fusion-Inducible Transgene Expression System for Neural Stem Cell-Based Antiglioma Gene Therapy

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Recent progress in neural stem cell- (NSC-) based tumor-targeted gene therapy showed that NSC vectors expressing an artificially engineered viral fusogenic protein, VSV-G H162R, could cause tumor cell death specifically under acidic tumor microenvironment by syncytia formation; however, the killing efficiency still had much room to improve. In the view that coexpression of another antitumoral gene with VSV-G can augment the bystander effect, a synthetic regulatory system that triggers transgene expression in a cell fusion-inducible manner has been proposed. Here we have developed a double-switch cell fusion-inducible transgene expression system (DoFIT) to drive transgene expression upon VSV-G-mediated NSC-glioma cell fusion. In this binary system, transgene expression is coregulated by a glioma-specific promoter and targeting sequences of a microRNA (miR) that is highly expressed in NSCs but lowly expressed in glioma cells. Thus, transgene expression is "switched off" by the miR in NSC vectors, but after cell fusion with glioma cells, the miR is diluted and loses its suppressive effect. Meanwhile, in the syncytia, transgene expression is "switched on" by the glioma-specific promoter. Our *in vitro* and *in vivo* experimental data show that DoFIT successfully abolishes luciferase reporter gene expression in NSC vectors but activates it specifically after VSV-G-mediated NSC-glioma cell fusion.

1. Introduction

Over the past decade significant progress has been made in development of neural stem cells (NSCs) as a novel gene delivery vector for antiglioma therapy [1]. NSCs are multipotent stem cells that give rise to the three fundamental neural lineages, neurons, astrocytes, and oligodendrocytes, throughout the central nervous system. When brain tumors are present, NSCs are capable of migrating through the brain parenchyma, by either existing or atypical routes, to home in on the tumor foci, including the original site and distant "satellite lesions" [1]. In animal models, the strong neoplastic tropism of NSCs has been extensively exploited for targeted delivery of therapeutic genes, such as the suicide gene thymidine kinase (TK), into brain tumors. These studies demonstrated that the genetically engineered NSC vectors can infiltrate the original tumor mass and chase down the advancing tumor satellites regardless of the injection location and its distance to the tumor and significantly suppress the tumor growth via overexpressing the therapeutic transgenes [2–6]. However, it was found that constant expression of the suicide gene TK from off-target NSCs caused significant cytotoxic effects on normal brain tissue [7]. Thus, it raises a safety concern that the use of these NSC vectors may deteriorate the situations of the patients.

To harness the cytotoxic effect of NSC vectors, we have previously engineered a novel antitumor gene, vesicular stomatitis virus G glycoprotein (VSV-G) H162R mutant, to eliminate tumor cells specifically under the acidic tumor microenvironment [8]. VSV-G is one class of the fusogenic

membrane glycoprotein (FMG) gene that can exert strong bystander killing effect via induction of multinucleated syncytia among the tumor cells [9]. However, the fusogenic capacity of the tumor acidosis-targeted H162R mutant is weaker than the wild-type; thus its killing efficacy still has much room to improve. Previous studies have shown that coexpression of suicide genes [10, 11], oncolytic viruses [12, 13], antitumoral chemokines [14], and cytokines [15, 16] with FMG can augment the tumor killing effects. Hence, we reason that induction of another transgene, such as the suicide gene TK, in the NSC/glioma syncytia could be a means to enhance the therapeutic efficacy of VSV-G-expressing NSC vectors.

To realize this while avoiding off-target side effects, it is desirable to restrict the transgene expression in a cell fusion-inducible manner. Double switching is originally a safety engineering practice that closes or opens both the positive and negative sides of an electrical circuit to prevent shock hazard in electric devices, which is an example of using redundancy to increase safety. Two recent studies have applied similar strategy to synthesize double-switch cellular circuits that couple transcriptional targeting and microRNA (miR) regulation to achieve precise control of transgene expression [7, 17]. In both studies, tumor-targeting promoters were used to activate transgene expression under on-target conditions, while highly expressed endogenous miRs were employed to knock down transgene expression under off-target conditions. The data showed that these double-switch expression cassettes obtained significantly higher specificities than single-promoter expression cassettes as the off-target "promoter leakage" was diminished by the "miR blockage."

Here we proposed a double-switch cell fusion-inducible transgene expression system (DoFIT) to enable therapeutic transgene induction upon VSV-G-mediated NSC/glioma syncytia formation. In this binary system, the transgene expression is coregulated by a glioma-specific promoter and targeting sequences of a miR that is highly expressed in NSCs but lowly expressed in glioma cells. Thus, the miR is employed to "switch off" the transgene expression in NSC vectors; however, after VSV-G-mediated cell fusion with glioma cells, the miR is diluted and its suppressive effect is gone. Meanwhile, in the syncytia, the glioma-specific promoter "switches on" the transgene expression. Our data showed that this combinatorial control using transcriptional targeting and miR regulation resulted in high transgene expression in glioma but negligible off-target expression in a mouse model.

2. Materials and Methods

2.1. Cell Culture. Human malignant glioma cell lines U87 and U251 were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin.

NSCs were derived from human induced pluripotent stem cells (iPSCs) using an adherent monoculture differentiation method as described previously [18]. In brief, iPSC colonies were detached from the 6-well cell-culture plate 7 days after plating by mechanical cutting. Then, iPSCs were then dissociated using TrypLE Express Dissociation Enzyme (Invitrogen) and plated onto a 0.1% gelatin-coated 6-well cell-culture plate at a density of 10^6 per well and cultured in NSC medium, which was a 1 : 1 mixture of DMEM/F12 (Invitrogen) supplemented with 2% B27 (Invitrogen), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 20 ng/mL EGF (Sigma-Aldrich), and 20 ng/mL bFGF (Invitrogen). Half of the cell-culture medium was changed every 2 days. After 7 days of differentiation, the cells reached 90% confluence and were split at ratio of 1 : 2. After 1 month of expansion, NSCs were derived from iPSCs. NSCs were digested using TrypLE for cell passage and subcultured at ratio of 1 : 2 twice weekly.

2.2. Plasmid Constructs. pGL4.11 (Promega) carrying the luc2P reporter gene was used as a starting backbone to construct double-switch transgene expression cassettes through multistep subcloning. Firstly, all promoters used in this study were placed upstream of the reporter gene. Secondly, 4 × microRNA targeting sequences (mirT) were designed to be perfectly complementary to the respective microRNA (in lowercase in Table 1) with 3 different linkers spacing each targeting sequence. The respective sense and antisense strands of the 4 × mirT oligonucleotides were phosphorylated, annealed, and then inserted downstream of the reporter gene. A control scramble targeting sequence (ScrT) of the same size was designed based on the lack of significant similarity to any known microRNA and subcloned into the same region (Table 1).

2.3. Luciferase Assay. Subconfluent cells in 48-well plate were transfected with plasmids encoding luciferase reporter gene at 400 ng per well, using 1.2 μL Fugene 6 (Roche) according to the manufacturer's protocol. After 24 h, the cells were lysed by freeze-thaw method and the supernatants were measured for luciferase activity using Luciferase Assay System (Promega) according to the manufacturer's instructions. All samples were assayed in triplicate.

2.4. MicroRNA qPCR. Small RNA was isolated using PureLink MicroRNA Isolation Kit (Invitrogen) and treated with TURBO DNA-free DNase (Ambion). Poly(A) tailing and cDNA synthesis of the DNAse-treated small RNA were performed using Ncode VILO MicroRNA cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. The forward primers for qRT-PCR analysis were designed based on entire known mature microRNA sequence, with additional 3 "A"s at the 3′ end to improve amplification specificity (Table 2). The reverse primer used was the Universal Primer in the EXPRESS SYBR GreenER MicroRNA qRT-PCR Kit (Invitrogen). 5S rRNA was selected as the internal reference gene for PCR quantification. To determine absolute copy number, a standard curve was generated using a synthetic LIN-4 RNA oligonucleotide.

qPCR was performed on iQ5 RT-PCR Detection System (BioRad). All reactions were run in triplicate.

Table 1: MicroRNA targeting (mirT) sequences.

mirT	Sequence
miR-199a-3p	
S1	5′-CTAGATAAtaaccaatgtgcagactactgtCGATtaaccaatgtgcagactactgt-3′
S2	5′-ACGCGTtaaccaatgtgcagactactgtTCACtaaccaatgtgcagactactgtGCATG-3′
AS1	5′-ACGCGTacagtagtctgcacattggttaATCGacagtagtctgcacattggttaTTAT-3′
AS2	5′-CacagtagtctgcacattggttaGTGAacagtagtctgcacattggtta-3′
ScrT	
S1	5′-CTAGAtaatttatgatctgcgcgtggagacgcccgattttatgatctgcgcgtggagacgcc-3′
S2	5′-acgcgttttatgatctgcgcgtggagacgcctcactttatgatctgcgcgtggagacgccGCATG-3′
AS1	5′-acgcgtggcgtctccacgcgcagatcataaaatcgggcgtctccacgcgcagatcataaattaT-3′
AS2	5′-Cggcgtctccacgcgcagatcataaagtgaggcgtctccacgcgcagatcataaa-3′

Table 2: MicroRNA qPCR primers.

MicroRNA	Primer sequence
hsa-miR-199a-5p	5′-CCCAGTGTTCAGACTACCTGTTCAAA-3′
hsa-miR-199a-3p	5′-ACAGTAGTCTGCACATTGGTTAAAA-3′
hsa-miR-214	5′-ACAGCAGGCACAGACAGGCAGTAAA-3′

2.5. Animal Experiment. Five BALB/c mice were anesthetized (132 μg/g ketamine and 8.8 μg/g xylazine) and received stereotactically guided injections of 10^6 U251 cells in 10 μL PBS through a 30-gauge Hamilton syringe into the right forebrain to allow glioma formation. After 1 week, 10^6 DoFIT-NSCs were injected into the left forebrain and glioma xenografts in the right forebrain, respectively. On the following 2 days, *in vivo* luciferase reporter gene expression levels were measured by the Xenogen IVIS-100 bioimaging system (Caliper).

2.6. Statistical Analysis. All data are represented as mean ± s.d. The statistical significance of differences was determined by Student's t-test or the two-factor analysis of variance analysis (ANOVA). A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. Selection of a Glioma-Specific Promoter. Firstly, we wish to select a promoter which has high activity in glioma cells but low activity in NSCs. Based on literatures, the astroglial lineage-specific promoter GFAP [7], the tumor-specific promoter Survivin [19], and the glioma-specific promoter HMGB2 [20] were chosen as candidates. The promoter activities were tested by luciferase assay in iPSC-derived NSC lines NSC1 and NSC2 and glioma cell lines U251 and U87. And the results were normalized by the activity of the strong universal promoter CMV (Figure 1(a)). The results showed that the HMGB2 promoter had the highest activity in glioma cell lines among all promoters. However, its averaged activity difference between NSC and glioma cell lines was merely 3.9-fold, thus requiring endogenous miR as an additional barrier to avoid the potential "promoter leakage."

3.2. Selection of an NSC-Specific miR. To select a miR which is expressed high in NSCs but low in glioma cells, we checked previous microRNA microarray data of the above NSC and glioma cell lines (unpublished data). As a result, hsa-miR-199a-5p, hsa-miR-199a-3p, and hsa-miR-214, all members of the miR-199a/214 cluster located on 1q24.3, were selected as candidates. miR qRT-PCR was employed to figure out their absolute expression levels in NSC1, NSC2, U87, and U251 (Figure 1(b)). The results showed that miR-199a-3p has the highest expression levels in NSC lines, up to 4.5 and 10 k copies per pg small RNA in NSC1 and NSC2, respectively. In addition, its specificity between NSC and glioma cell lines is also the highest among all candidate miRs, more than 682-fold. In both U87 and U251, its expression levels are much less than 100 copies per pg small RNA, which is insufficient to abolish transgene expression according to previous report [21]. Therefore, miR-199a-3p was chosen as the optimal suppressor of transgene expression within NSC vectors for glioma targeting.

3.3. Double-Switch Glioma-Specific Transgene Expression Cassette. Glioma-specific transgene expression cassette under combinatory control of HMGB2 promoter and 4 × miR-199a-3p targeting sequences (pHMGB2-mir199a3pT) was constructed. Moreover, to rule out the possibility of less favorable transcription caused by introduction of a long repeat sequence into the 3′-UTR, a control construct pHMGB2-ScrT with a mismatched miR targeting sequence of the same length in the 3′-UTR was generated (Figure 2(a)). The original pHMGB2 construct and the above 2 new constructs were tested by luciferase assay in U251 and NSC1. The results indicated that, compared to pHMGB2-ScrT, pHMGB2-mir199a3pT obtains a reduction of luciferase activity up to 98.7% in NSC1. Additionally, the combinatorially regulated construct showed no significant decrease

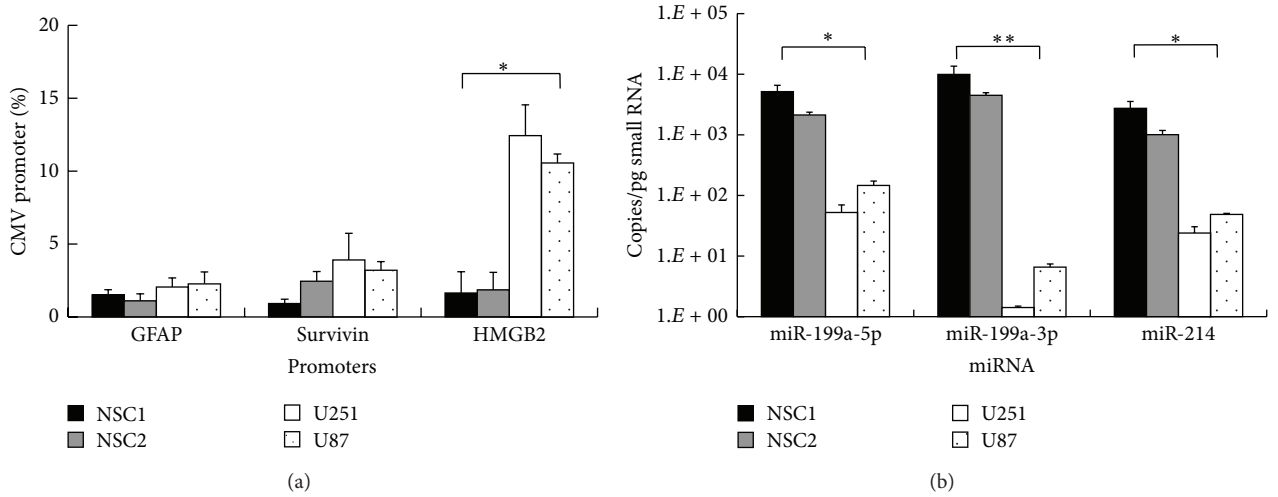


FIGURE 1: Selection of glioma-specific promoter and NSC-specific miR. (a) Promoter activities of the lineage-specific promoter GFAP, tumor-specific promoter Survivin, and glioma-specific promoter HMGB2 compared to the strong universal promoter CMV in different NSC and glioma cell lines are quantified by luciferase assays. (b) Absolute expression levels of miR-199a/214 cluster members miR-199a-5p, miR-199a-3p, and miR-214 in different NSC and glioma cell lines are quantified by qPCR. miR copy numbers were calculated based on a standard curve generated using a synthetic LIN-4 RNA oligonucleotide. Error bars: s.d. $^{*}P < 0.05$, $^{**}P < 0.01$.

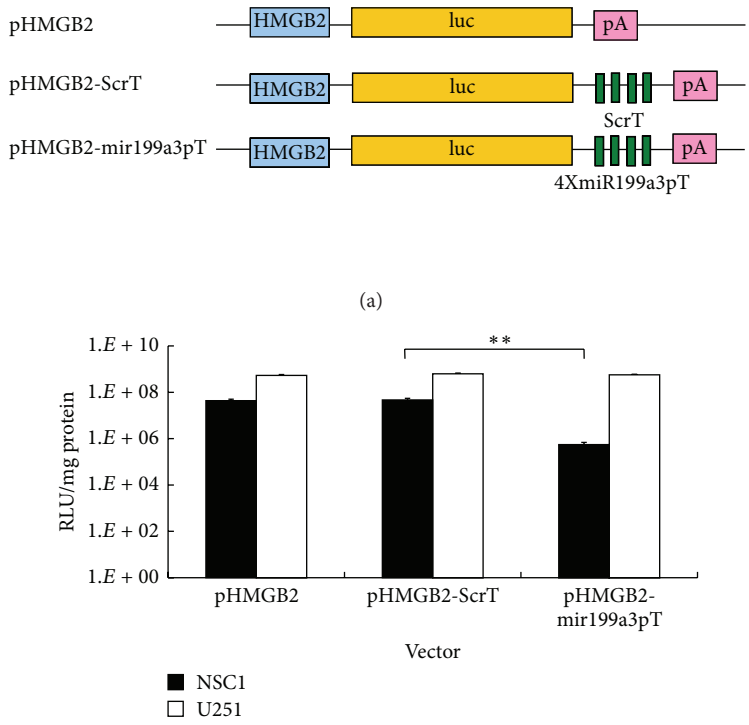


FIGURE 2: Combinatory effect of optHRP and miR-199a-5p on transgene regulation. (a) Schematic representation of the combinatorial expression cassettes containing the HMGB2 promoter and miRNA target sequences. HMGB2, high mobility group box 2 gene promoter; luc, luciferase reporter gene; miR-199a-3p and scramble target sequences as detailed in Table 1 were inserted into 3′-UTR, respectively; pA, polyA signal. (b) Transgene expression levels of different expression cassettes within NSC1 and U251 cell lines are quantified by luciferase assays. Error bars: s.d. $^{*}P < 0.05$, $^{**}P < 0.01$.

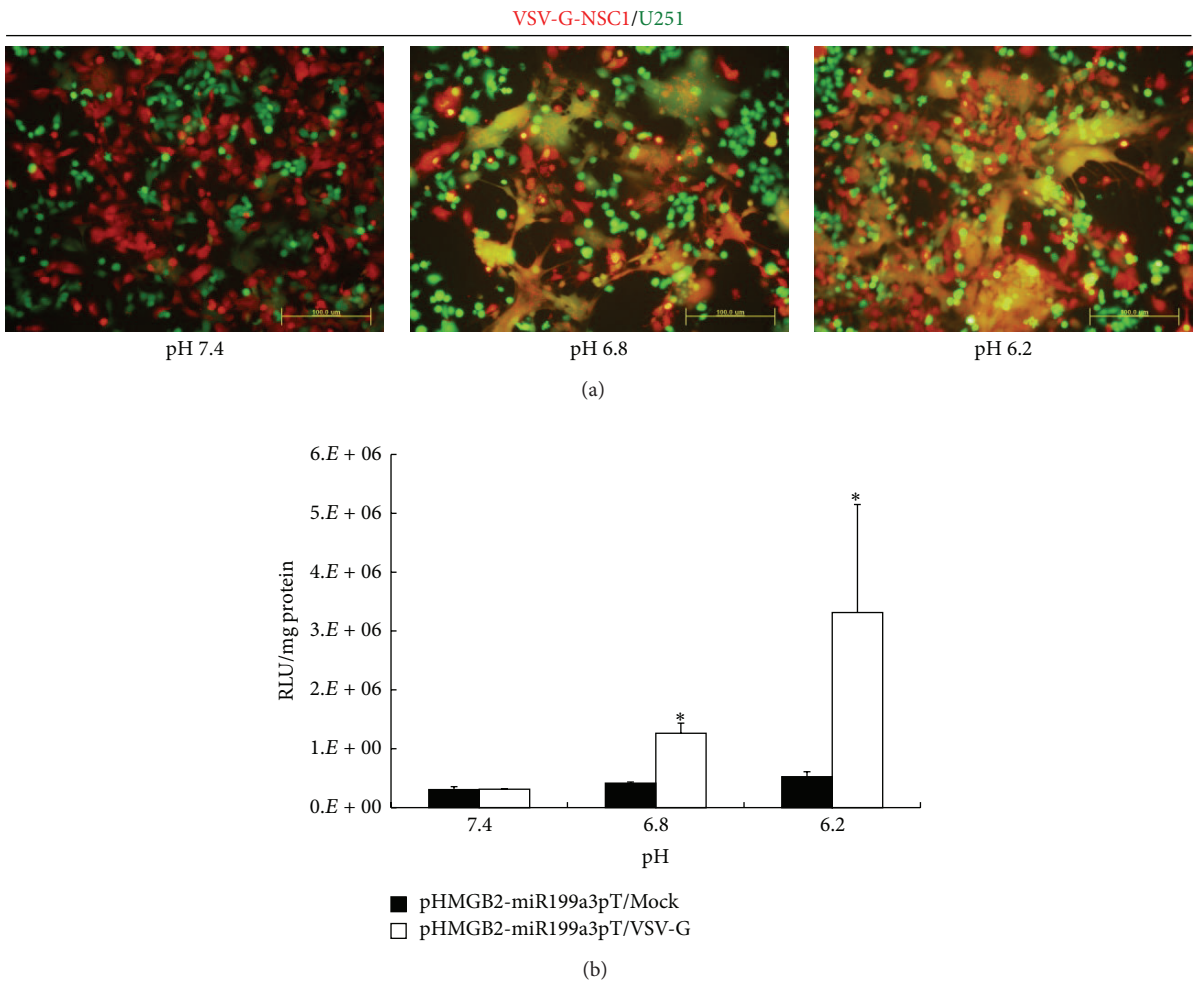


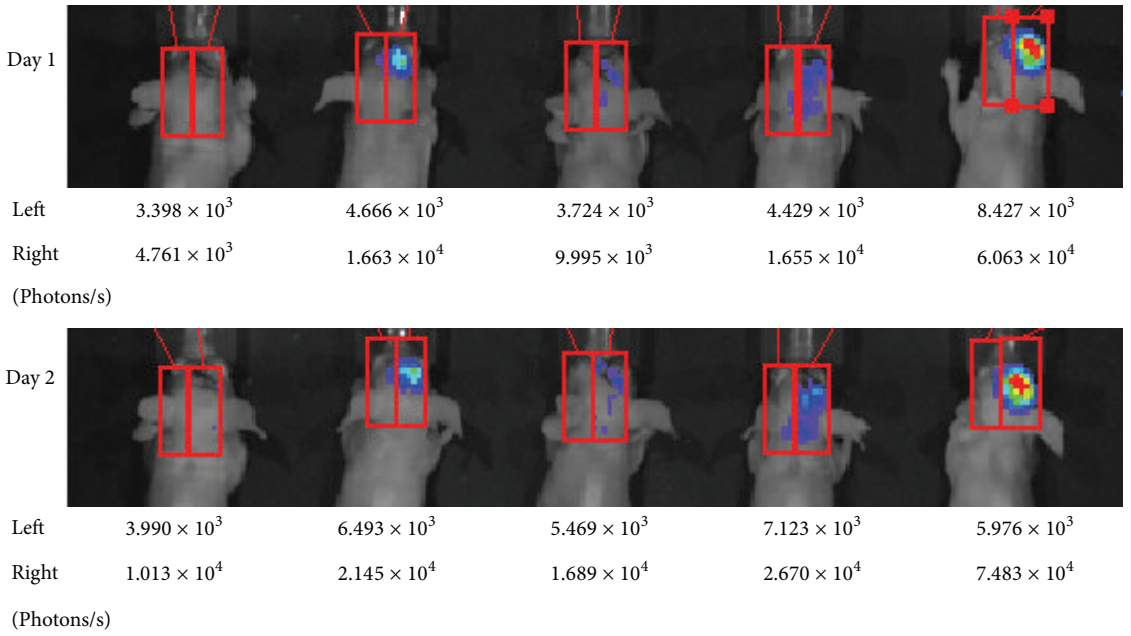
Figure 3: DoFIT mediates cell fusion-inducible transgene expression *in vitro*. (a) Low pH-dependent cell fusion between VSV-G-expressing NSC1 and U251 is examined by dual-color syncytia formation assays. (b) Transgene expression levels of cocultures between DoFIT-NSCs and U251 under different pH conditions are quantified by luciferase assays. Error bars: s.d. $^{*}P < 0.05$, $^{**}P < 0.01$.

of transgene expression in U251 compared to other constructs (Figure 2(b)). Therefore, the double-switch construct pHMGB2-mir199a3pT was demonstrated to have a great inhibition on transgene expression by miR regulation in NSCs without compromising the promoter induction in glioma cells.

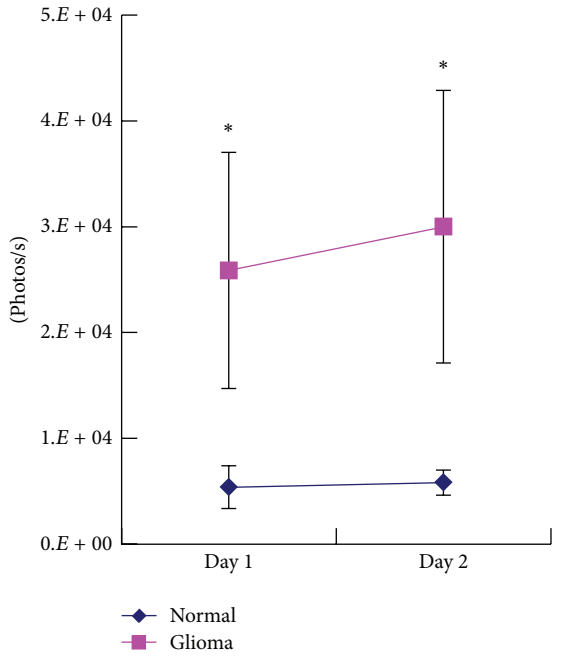
3.4. DoFIT Mediates Cell Fusion-Inducible Transgene Expression In Vitro. Firstly, a dual-color syncytium formation assay was performed to test the ability of VSV-G to cause cell fusion between NSCs and glioma cells. NSC1 was transfected with pVSV-G, a plasmid encoding the VSV-G mutant H162R, and stained with the red-orange dye DiL and then cocultured with U251 at a ratio of 1 : 1 at pH 7.4, 6.8, and 6.2, respectively. pH 7.4 represents the normal physiological pH, pH 6.8 represents the typical acidic tumor extracellular pH (pH_e), and pH 6.2 represents an optimal low pH that favors the fusogenic function of VSV-G. The results showed that VSV-G could mediate efficient cell fusion between NSCs and glioma cells at both pH 6.8 and 6.2 but not at the neutral pH 7.4 (Figure 3(a)).

Secondly, to examine whether pHMGB2-mir199a3pT can mediate cell fusion-inducible transgene expression *in vitro*, cell fusion luciferase assay was applied to NSC1/U251 coculture. NSC1 was transfected with pHMGB2-mir199a3pT together with pVSV-G or a Mock vector. Then the transfected cells were cocultured with U251 at a ratio of 1 : 1 under pH 7.4, 6.8, and 6.2, respectively. After 24 h, luciferase activity was measured. The results indicated that, at pH 7.4, no transgene expression was induced. At pH 6.8, only pHMGB2-mir199a3pT/VSV-G was induced. The induction ratio was 3-fold compared to pHMGB2-mir199a3pT/Mock. At pH 6.2, both pHMGB2-mir199a3pT/VSV-G and pHMGB2-mir199a3pT/Mock were induced (Figure 3(b)). Thus, it demonstrated that DoFIT can mediate tumor pH_e-dependent cell fusion-inducible transgene expression *in vitro*.

3.5. DoFIT Mediates Glioma Site-Targeted Transgene Expression In Vivo. To further examine whether DoFIT can mediate glioma-targeted transgene expression *in vivo*, an animal study was performed using an orthotopic mouse model of



(a)



(b)

Figure 4: DoFIT mediates NSC-delivered glioma-specific transgene expression *in vivo*. (a) Bioluminescent images showing transgene expression in the glioma-bearing mice. U251 cells are inoculated into the right forebrains of the mice. After the gliomas develop, DoFIT-NSCs are inoculated into both the left and the right forebrains of the mice. Bioluminescent images are taken on day 1 and day 2 after NSC inoculation. The luminescent readings (photons/sec) of both left and right forebrains are indicated below the images. (b) Line graph showing trends of the averaged transgene expression levels in the glioma sites (right forebrain) and the normal sites (left forebrain) over day 1 and day 2 after NSC inoculation. Statistical significance of glioma sites versus normal sites at each time point was calculated by Student's t-test. Error bars: s.d. $^{*}P < 0.05$.

glioma. In brief, U251 was implanted into the right forebrain of BALB/c mice in advance to allow glioma formation. After 1 week, 10^6 DoFIT-transfected NSCs were injected into the control left forebrains and glioma-xenografted right forebrains, respectively. On the following 2 days, luciferase reporter gene expression levels in the mice were measured using a live animal imaging platform. The results showed that DoFIT-NSCs could mediate transgene expression in the glioma-xenografted right forebrains but remained silenced in the control left forebrains (Figure 4(a)). Moreover, the average luminescent level in the right forebrains elevated by 20% from day 1 to day 2 (Figure 4(b)), which was probably due to more NSC arrival into the glioma sites and more cell fusion events.

4. Discussion

We have shown here a combinatorial transgene expression system DoFIT, which couples glioma-specific promoter HMGB2 and miR-199a-3p regulation to enable high level of transgene activation upon VSV-G-mediated NSC/glioma cell fusion but remains silenced in NSC vectors under off-target conditions.

Annually, there are approximately 189,000 new brain tumor cases diagnosed and 142,000 deaths documented worldwide, 80% of which are due to gliomas [22]. In spite of the relatively low incidence of glioma, the highly lethal nature of this cancer results in a median survival time of 15 months for patients with glioblastoma multiforme, grade IV glioma [23]. Despite surgical resection, combination radiation therapy, and chemotherapy, the cancer usually relapses due to extensive invasion of tumor cells into the normal brain parenchyma and acquisition of therapeutic resistance [24]. Hence, there is an urgent need to develop NSC-based antiglioma gene therapy that is able to target multiple invasive tumor foci intracranially, thus improving the therapeutic efficacy for this deadly disease.

The great potential of NSCs in cancer gene therapy highlights the importance of a robust, reliable source for the large scale, standardized production of human NSCs that meets the requirements of good clinical practice. The use of human iPSCs has provided an accessible and stable source to produce unlimited amounts of NSCs for cell-based therapies [4]. Also, iPSC-based therapeutic approaches bypass the sensitive ethical issue of human embryonic stem cells [25] and the safety concern of immune rejection by allogeneic transplantation. In this study, two iPSC-derived NSC lines were generated and could be genetically manipulated for the test of our system *in vitro* and *in vivo*.

Remarkably, our study has demonstrated that the intrinsic differences in endogenous miR expression patterns can be exploited to segregate the transgene expression between closely related cellular lineages. In this study, several glioma-specific promoters were tested. However, none of their specificities between NSC and glioma cell lines are sufficiently satisfactory, probably due to some similar characteristics shared between NSCs and glioma cells. Fortunately, substantial studies from different groups have demonstrated the feasibility of employing endogenous miRNA to inhibit transgene expression in a cell type-specific manner [7, 21, 26, 27]. Similarly, by incorporating targeting sequences of a highly expressed endogenous miR in NSCs into the transgene expression cassette, the selectivity between NSC and glioma cell lines increases by approximately 100-fold, indicating a strong capacity of endogenous miR regulation to minimize the potential off-target transgene expression caused by "promoter leakage." Consistent with previous study, our data show that miR lower than 100 copies per pg small RNA has no significant suppressive effects on transgene expression [21].

More importantly, the animal experiment using an orthotopic brain tumor mice model demonstrated that our combinatorial system achieved very robust biphase transgene expression *in vivo*. The DoFIT-NSCs injected into normal forebrains displayed undetectable luciferase gene expression; meanwhile, vectors in glioma sites exhibited strong luminescent signals, and the signals even increased on the next day. This is reasonable as our previous studies showed that there would be more NSC vectors migrating into the tumor sites and more VSV-G-mediated cell fusion occurred [8]. Hence, it proves that our binary regulatory system is capable of abolishing off-target transgene expression without compromising the on-target expression *in vivo*.

5. Conclusion

In summary, our work demonstrates an inducible, non-leaky transgene expression system which functions within NSC-based gene delivery vectors for VSV-G-mediated NSC/glioma cell fusion-inducible transgene expression. Our data exhibit that it is able to trigger robust luciferase reporter gene expression upon NSC/glioma cell fusion at tumor pH_e *in vitro* and at glioma sites *in vivo*. Most importantly, a negligible transgene expression level in off-target region is observed, indicating the increased safety by applying an additional barrier of miR regulation. Further refinement of this system may lead to the development of optimal cell-based gene delivery vector to target malignant gliomas.

Conflict of Interests

All authors have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yumei Luo and Detu Zhu contributed equally to this work.

Acknowledgments

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Research Article

Human Mesenchymal Stromal Cells Transplantation May Enhance or Inhibit 4T1 Murine Breast Adenocarcinoma through Different Approaches

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The use of Mesenchymal Stromal Cells (MSCs) aiming to treat cancer has shown very contradictory results. In an attempt to clarify the contradictory results reported in the literature and the possible role of human fallopian tube Mesenchymal Stromal Cells (htMSCs) against breast cancer, the aim of this study was to evaluate the clinical effect of htMSCs in murine mammary adenocarcinoma using two different approaches: (1) coinjections of htMSCs and 4T1 murine tumor cell lineage and (2) injections of htMSCs in mice at the initial stage of mammary adenocarcinoma development. Coinjected animals had a more severe course of the disease and a reduced survival, while tumor-bearing animals treated with 2 intraperitoneal injections of 10^6 htMSCs showed significantly reduced tumor growth and increased lifespan as compared with control animals. Coculture of htMSCs and 4T1 tumor cells revealed an increase in IL-8 and MCP-1 and decreased VEGF production. For the first time, we show that MSCs isolated from a single source and donor when injected in the same animal model and tumor can lead to opposite results depending on the experimental protocol. Also, our results demonstrated that htMSCs can have an inhibitory effect on the development of murine mammary adenocarcinoma.

1. Introduction

Mesenchymal Stromal Cells (MSCs) are undifferentiated multipotent cells with potential for self-renewal and differentiation into several distinct cell lineages [1]. They are composed of a heterogeneous population of cells, constituting a reservoir within the connective tissue of most organs involved in the maintenance and repair of tissues throughout the course of life. MSCs present a similar profile of cell surface receptor expression, although they are defined by their functional properties rather than by marker expression.

MSCs can be isolated from different tissues [2–6]. We have previously described the presence of MSCs in human fallopian tube (human tube Mesenchymal Stromal Cells—htMSCs) that were able to differentiate into cartilage, muscle, bone, and adipose cell lineages *in vitro* [6]. Moreover, htMSCs were able to enhance bone maturation *in vivo* in a xenotransplanted model, suggesting that in the future they might be used to treat bone diseases, such as osteoporosis [7]. Breast cancer, the leading form of cancer in women and the second leading cause of cancer mortality worldwide, is a very complex disease and treatment protocols are continually changing [8].

Previous studies aiming to analyze the clinical effect of MSCs in cancer have shown very discrepant results, enhancing [9–11] or inhibiting tumor growth [12–14] in animal models which were injected with different MSCs and with different tumor cell lines. Klopp and colleagues [15] published an important review on the discrepant results warning that

experiments made with different methodologies cannot be compared. For example, different protocols were reported for cell-injections (coinjection, systemically, subcutaneously, or intraperitoneally), number and origin (human or murine) of injected MSCs, and injection's schedule of MSCs in each model (before, during, or after the establishment of primary tumor).

One of the best known models for breast cancer studies is the 4T1 murine mammary tumor cell line. Originally isolated by Miller et al. [16], the 4T1 cell line inoculated at the mammary fat pad presents a high tendency to metastasize to several organs such as lungs, liver, brain, and bone, which are also involved in human breast cancer [17, 18].

Muehlberg et al. [19] showed that murine adipocyte stem cells (mASCs) promote tumor growth *in vivo* when coinjected with 4T1 mammospheres or when systemically injected 12 hours after 4T1 local injection. Altman et al. [20] also showed that human ASCs injected intravenously or subcutaneously coinjected with 4T1 cell line are directed to the tumor site, increasing its volume. But the observed results were significant only in the subcutaneously coinjected group.

In an attempt to clarify these controversial results, the aim of this study was to assess the effect of htMSCs on 4T1 murine breast carcinoma development, using two different approaches: (1) coinjection of htMSCs and tumor cells and (2) injection of htMSCs in tumor-bearing animals.

2. Materials and Methods

2.1. Human Tube MSCs Culture Establishment. Four human fallopian tubes (hFTs) were obtained from hysterectomy or tubal ligation/resection samples collected during the proliferative phase from fertile women. Informed consent was obtained from each patient and approval granted by the Biosciences Institute Ethics Committee of the University of São Paulo.

Cell lines were obtained as described previously [6], with modifications. hFTs samples were washed twice in phosphate saline buffer (PBS, Life Technologies, Carlsbad, CA), finely minced with a scalpel, and put inside a 50 mL conical tube. Then, 5 mL of 0.1% collagenase (Sigma-Aldrich) diluted in PBS was added and samples were incubated for 15 minutes, at 37°C, in a water bath. After the first incubation, 5 mL of pure DMEM/F-12 (Life Technologies) was added and gently mixed. Shortly thereafter, 10 mL of pure TripLE Express (Invitrogen, Carlsbad, CA) was added, gently mixed, and incubated for 15 minutes, at 37°C, in a water bath. Subsequently, supernatant was removed with a sterile Pasteur pipette; cells were washed once with 20 mL of DMEM/F-12 supplemented with 10% fetal bovine serum (FBS, Life Technologies) and pelleted by centrifugation at 400 g for 5 minutes at room temperature. Cells were then plated in plastic flasks (25 cm^2, Corning, New York, USA) in DMEM/F-12 Media-GlutaMAX-I (5 mL) supplemented with 10% FBS, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 1% nonessential amino acids solution (all Invitrogen) and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. The culture medium was routinely replaced twice a week thereafter.

In the third passage, htMSCs were characterized by their differentiation potential and superficial markers (flow cytometry), as described below.

One lineage was randomly chosen for *in vivo* experiments. The other 3 lineages were analyzed *in vitro*.

2.2. MSCs Characterization. To evaluate the properties of htMSCs differentiation, adherent cells underwent *in vitro* adipogenic, chondrogenic, and osteogenic differentiation using Life Technologies Stem Prodifferentiation medium kits (A1007101, A1007001, and A1007201), as indicated by the manufacturer. Flow cytometric analysis was provided for antihuman antibodies CD14 (VMRD Inc., Pullman, WA), CD29-PE-Cy5, CD31-PE, CD44-FITC, CD45-FITC, CD73-PE, CD90-R-PE, human leukocyte antigens- (HLA-) ABC-FITC and HLA-DR-R-PE (Becton Dickinson), and SH4 (kindly provided by Dr. Irina Kerkis, Butantan Institute, São Paulo, Brazil). Unconjugated markers were reacted with anti-mouse PE secondary antibody (Guava Technologies). All methods were described before [6, 7].

2.3. Tumor Cell Line. Murine mammary adenocarcinoma cells (4T1 cell line), obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were expanded in RPMI-1640 medium (pH 7.2), supplemented with 10% FBS, 10 mM HEPES [N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)], and 24 mM $NaHCO_3$ (all from Life Technologies).

2.4. Animals. Fifty-one 8-week-old immunocompetent BALB/c female mice, from Inbred Mice Bioterium of Institute of Biomedical Sciences of Universidade de São Paulo (ICB/USP) and from Centre for Development of Experimental Models for Medicine and Biology (CEDEME/UNIFESP), were used. This research, which involves the use of murine tumor cells and human stromal cells in murine animal models, was approved by the Research Ethics Committee of the Federal University of São Paulo. For the experimental groups, the animals were divided into subgroups of 6 or 7 animals.

2.5. In Vivo Experimental Design

2.5.1. Coinjection of MSCs and 4T1 Tumor Cell Lineage. For this experiment, 12 BALB/c mice were divided into 2 groups of 6 animals: G1, coinjected in the mammary fat pad with 10^6 htMSCs and 10^4 4T1, and G2, untreated control group, injected in the mammary fat pad with 10^4 4T1 (Figure 1(a)).

2.5.2. Injection of htMSCs in Tumor-Bearing Mice. For this experiment, 21 BALB/c mice were firstly injected with 10^4 4T1 cells into the mammary fat pad and afterwards the animals were divided into 3 groups of 7 animals per group: G3, treated with 1 intraperitoneal injection of 10^6 htMSCs, 7 days after

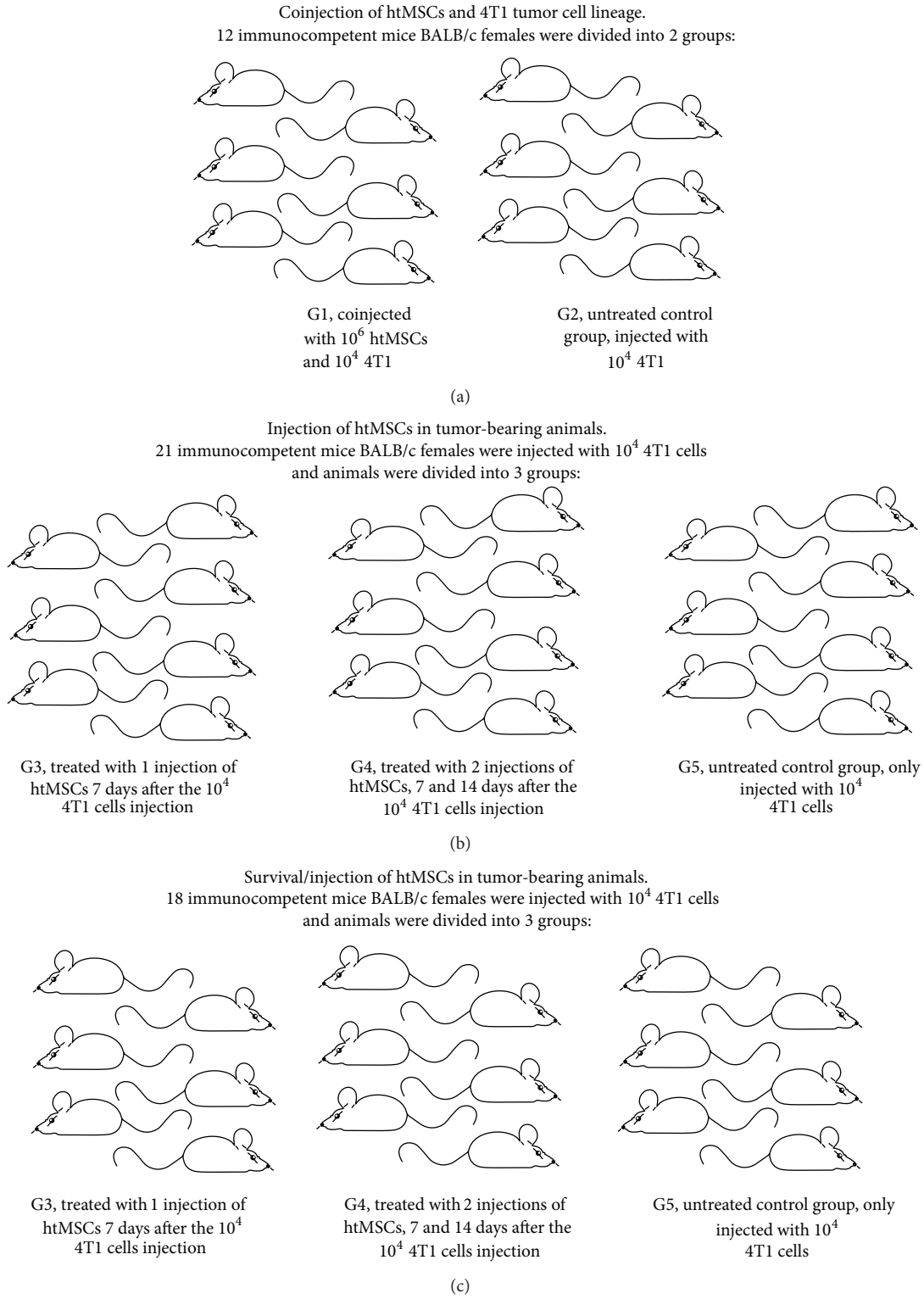


Figure 1: Experimental design, where (a) represents the coinjected group; (b) and (c) represent tumor-bearing animals injected with htMSCs.

the inoculation of 4T1 cells; G4, treated with 2 intraperitoneal injections of 10^6 htMSCs, 7 and 14 days after the inoculation of 4T1 cells; G5, untreated control group, only injected with 4T1 cells (Figure 1(b)).

2.5.3. Survival. For survival analysis, 18 BALB/c mice were divided into 3 groups of 6 animals, treated as described in Section 2.5.2 and monitored daily until natural death (Figure 1(c)).

The protocol for tumor 4T1 cells inoculation (10^4 cells injected into the mammary fat pad) was previously standardized (data not shown). In these conditions, primary tumors were visible in about 7 days. The MSCs dose of 10^6 cells apparently was well tolerated after intraperitoneal or intravenous injections in mice, with no visible changes in animals.

2.6. Primary Tumor Growth. During the *in vivo* experiments and postmortem, primary tumor volumes were measured with a mechanical caliper every three days, and the tumor volume was calculated using the formula [(higher value)(smaller value)^2] $\times$ 0.52.

2.7. Postmortem Animals Examination. Three coinjected animals were analyzed right after natural death. The remaining mice were analyzed right after euthanasia in the CO_2 gas chamber. Primary tumors were collected and the presence of intraperitoneal metastatic tumors was registered by digital images.

2.8. Tissues Histology. Two primary tumors and lungs from each experimental group were fixed in 10% formalin (diluted in 1X PBS) for one week at room temperature and paraffin-embedded. For histological analysis, slides (5 μm thick) were cut and dyed with Hematoxylin-Eosin. Additionally, primary tumors were analyzed for the presence of human cells through the analysis of the specific human nuclei lamin A/C (anti-lamin A + C, Abcam Inc., Cambridge, MA, USA).

2.9. Pulmonary Nodules and Inflammation Analysis. For this analysis, the lungs of all animals were removed and dyed with Bouin's Solution. After 48 h, Bouin's Solution was removed and replaced by 10% formalin (diluted in 1X PBS). Digital images were obtained from each organ and pulmonary metastases/nodules were counted in a stereomicroscope (Nikon, Tokyo, Japan). Afterwards, two lungs from each experimental group were paraffin-embedded for histological analysis (Hematoxylin-Eosin). The levels of pulmonary inflammation and tumor tissues (metastasis) were analyzed measuring the free area, that is, the tissue-free space, of each lung (tool available in the software NIS Elements Nikon AR).

2.10. Immunohistochemistry. For immunohistochemistry analysis, 3 μm sections of primary tumor specimens were deparaffinized, rehydrated, and incubated in 6% aqueous hydrogen peroxide for 30 min to quench endogenous peroxidase activity. The slides were heated to 95°C for 45 min in EDTA buffer for antigen retrieval and treated with 0.5% pepsin, pH 1.8 for 30 min at 37°C. The sections were incubated with a human specific anti-lamin A + C antibody (ab108595, Abcam, Cambridge, UK). ENVISION HRP system (Dako, Carpinteria, CA, USA) was used to detect the nuclear lamin proteins of the htMSCs. Samples were lightly counterstained with Mayer's hematoxylin, dehydrated, and mounted with glass coverslips and xylene-based mounting medium. Non-immune serum was used as negative control, and human-origin cartilage micromass, originated by htMSCs chondrogenic differentiation *in vitro* [6], was used as positive control.

2.11. Identification of Cytokines Released by htMSCs In Vitro. Aiming to verify the production of cytokines released by htMSCs when they are in the tumor microenvironment, we cultivated htMSCs with 4T1 tumor cells *in vitro* at the ratio 1 : 1 (2×10^5 cells) in 6-well plates (3 mL of culture media per well). We used media DMEM/F-12 supplemented with 20% of FBS and 100 IU/mL penicillin and 100 IU/mL streptomycin (all Life Technologies). Cells were physically separated by culture inserts (0.4 μm PET, Millipore, Darmstadt, Germany) and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 48 h.

The Bio-Plex Pro Human Cytokine 27-Plex Immunoassay Panel (Bio-Rad, Hercules, CA, USA, number M50-0KCAF0Y) includes 27 magnetic bead-based assays to measure FGF basic, eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-s (MCAF), MIP-1alpha, MIP-1beta, PDGF-BB, RANTES, TNF-alpha, and VEGF. Supernatant was harvested and processed according to the Bio-Assays-Plex Pro manufacturer's instructions. For this experiment, we used three lineages of different htMSCs, each one in triplicate.

2.12. Statistical Analyses. Statistical analyses were done by ANOVA test with Tukey's test track (by Microsoft Excel 2010) and by the Software Prism 5 for the survival analysis.

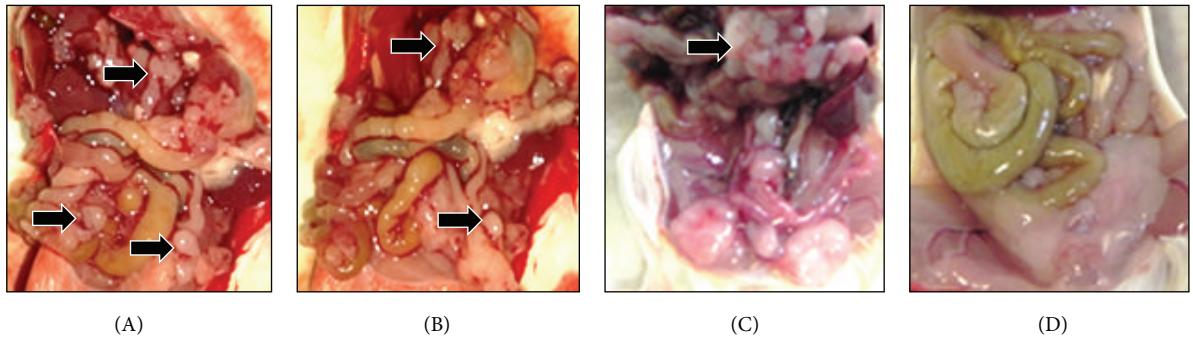
3. Results

3.1. htMSCs Characterization. htMSCs used in this experiment differentiated in adipogenic, chondrogenic, and osteogenic tissues *in vitro* and presented as well the expected profile of surface markers by cytometry, as described before [6, 7] (data not shown).

3.2. Coinjection of htMSCs and 4T1 Tumor Cell Lineage

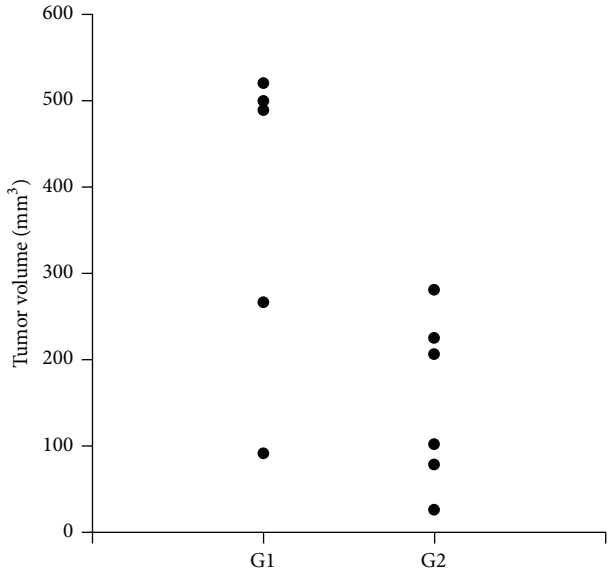
3.2.1. Tumor Growth and Inflammation Analysis. All animals developed primary tumors. However, some coinjected animals (G1) survived only 15 days, and the necropsies realized in all animals at day 15 showed many tumor masses in the abdominal and thoracic region of G1 group, while untreated animals (G2) presented only primary tumor growth and no visible nodules in the abdominal/thoracic region (Figure 2(a)). Furthermore, the primary tumor volume was significantly increased in 4 of 6 animals of the coinjected group (Figure 2(b)).

Macroscopic lung analysis showed preserved organs but possible tumor masses near the trachea in all coinjected animals. Microscopic analysis of the lungs showed no visible tumor nodules and primary tumors with similar histology in both groups. Although no differences in lungs were evident between the groups in macro- and microscope analysis, when the size of tissue-free areas in the lungs was compared, a reduction of 40% in the coinjected group was observed (Figure 3). These results suggest that htMSCs coinjected with 4T1 breast carcinoma cells exacerbate primary tumor growth,



(A) (B) (C) (D)

(a)



Tumor volume (mm^3)		
Mice	G1 (coinjected)	G2 (control/untreated)
1	266.24	101.92
2	499.2	280.28
3	520	25.74
4	488.41	205.92
5	91	224.64
6	Not provided (irregular)	78
Average	372.97	152.75

(b)

Figure 2: Coinjected group necropsy and tumor growth analysis. (a) Abdominal view of coinjected animals (A, B, and C) and untreated control (D), on day 15. Arrows indicate probable tumor nodules. One representative animal of each group is shown. (b) Primary tumor volume at death (day 15), showing that coinjected animals (G1) presented, on average, primary tumor volumes about 2.4x higher than the untreated group (G2). Animals are represented individually.

reduce the inflammation-free area of the lungs, and facilitate abdominal metastasis development.

3.3. Injection of htMSCs in Tumor-Bearing Mice. All groups (G3, G4, and G5) were analyzed 20 days after tumor cells inoculation. None or just few abdominal nodules were found in some animals of the 3 groups, but no lung metastatic nodules were macroscopically visible in any group (Figure 4). Group G4, treated with 2 injections of htMSCs, showed a lower number of microscopic pulmonary nodules on the 20th day when compared to group G3 and the untreated control group (G5), as expected, since the animals were in better physical conditions. But it is important to point that all animals (G3, G4, and G5 groups) presented microscopically visible tumors 20 days after the onset of the experiment. Differences between groups were also evident when the tissue-free lung area was compared, showing that treatment of mice with 2 doses of htMSCs restored the lung areas free of inflammation and metastasis, as compared to normal mice (Figure 4(b)).

3.4. Immunohistochemistry. Human nuclei were found neither in primary tumor (Figure 5) nor in lung metastasis (data not shown) in animals from any group, including the coinjected group G4, 15 days after tumor cell inoculation. This result suggests that tumors were formed exclusively by murine tumor cells, and the injected htMSCs were not present at the tumor microenvironment in the evaluated timepoint.

3.5. Survival and Tumor Growth. Animals in the control group (G5) died from day 30 to day 35. The group treated with only 1 htMSCs injection (G3) started dying on day 31, and at day 37, 85% of animals had already died. On the contrary, only 15% of animals treated with 2 htMSCs injections (G4)

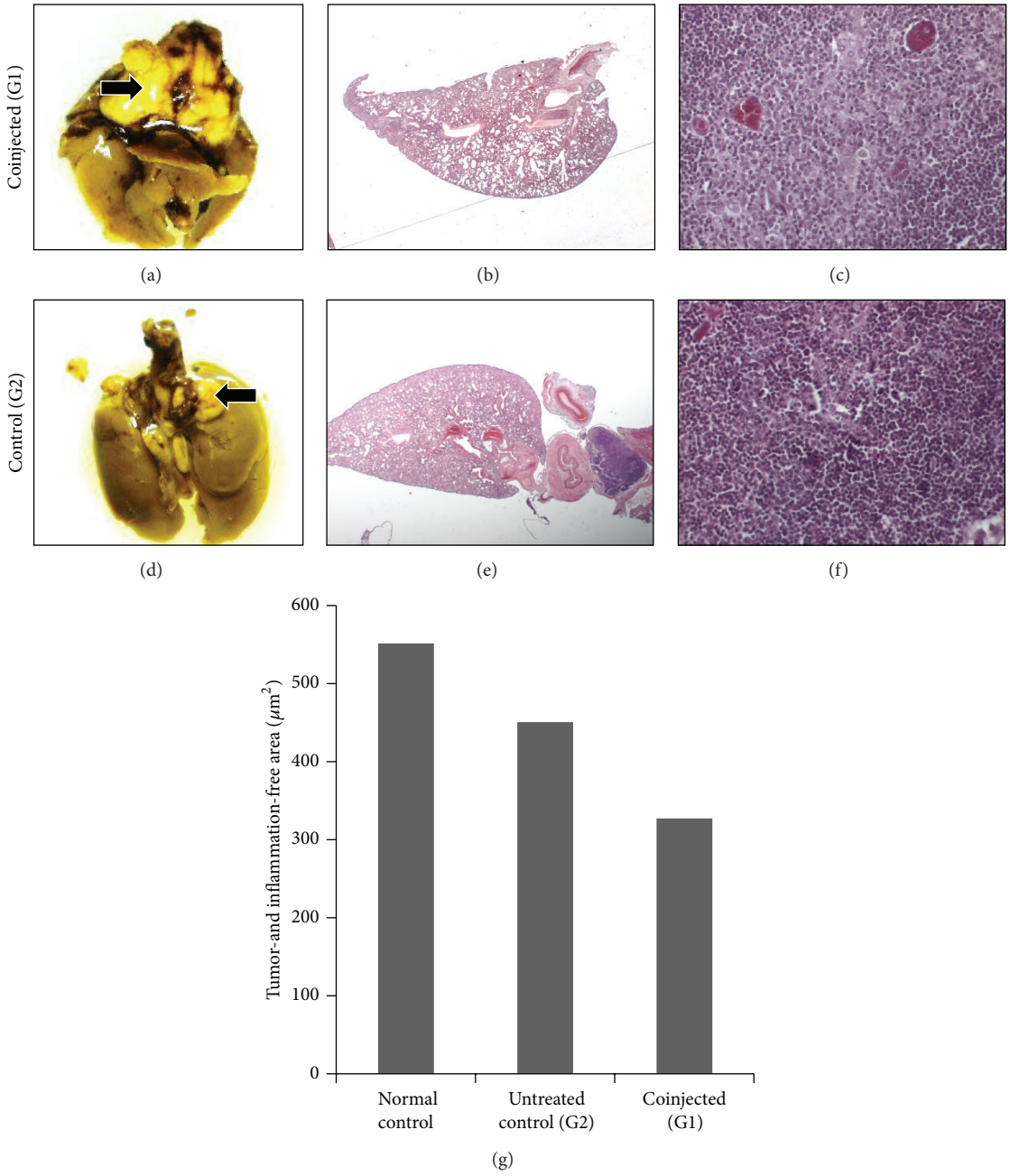


FIGURE 3: Tumor analysis of G1 and G2 groups. ((a) and (d)) Lungs macroscopic view; ((b) and (e)) lungs microscopic view (2.5x); ((c) and (f)) primary tumors microscopic view (40x). One representative animal of each group is shown. (g) Tissue-free measurement in lungs, showing that coinjected animals presented an increase in inflammation areas in the lungs, despite not having died from respiratory insufficiency. Arrows indicate possible tumor masses near trachea in (a) and (d).

had died at day 38, which represent a highly statistically significant difference as compared to the control group ($^*P$ value = 0.0001) (Figure 6). Furthermore, 2 htMSCs injections reduced primary tumor volumes in G4 group as compared to the other 2 groups (Figure 7(a)). At least until day 23 after tumor inoculation, average primary tumor volume of the G4 group was significantly reduced compared to untreated control group (G5). Statistically significant differences were lost in later measurements (Figure 7(b)).

3.6. Identification of Cytokines Released by htMSCs In Vitro. Murine tumor cells and htMSCs were cultivated separately (controls) in complete medium for 48 h and human cytokines were analyzed in the culture supernatant. As expected, human cytokines were not detected in the control murine cells supernatant. In the culture supernatant of control htMSCs, only 4 cytokines were detected among the 27 analyzed by the assay: IL-6, IL-8, MCP1, and VEGF. After cocultivation with no direct contact of both cell lines (using a transwell),

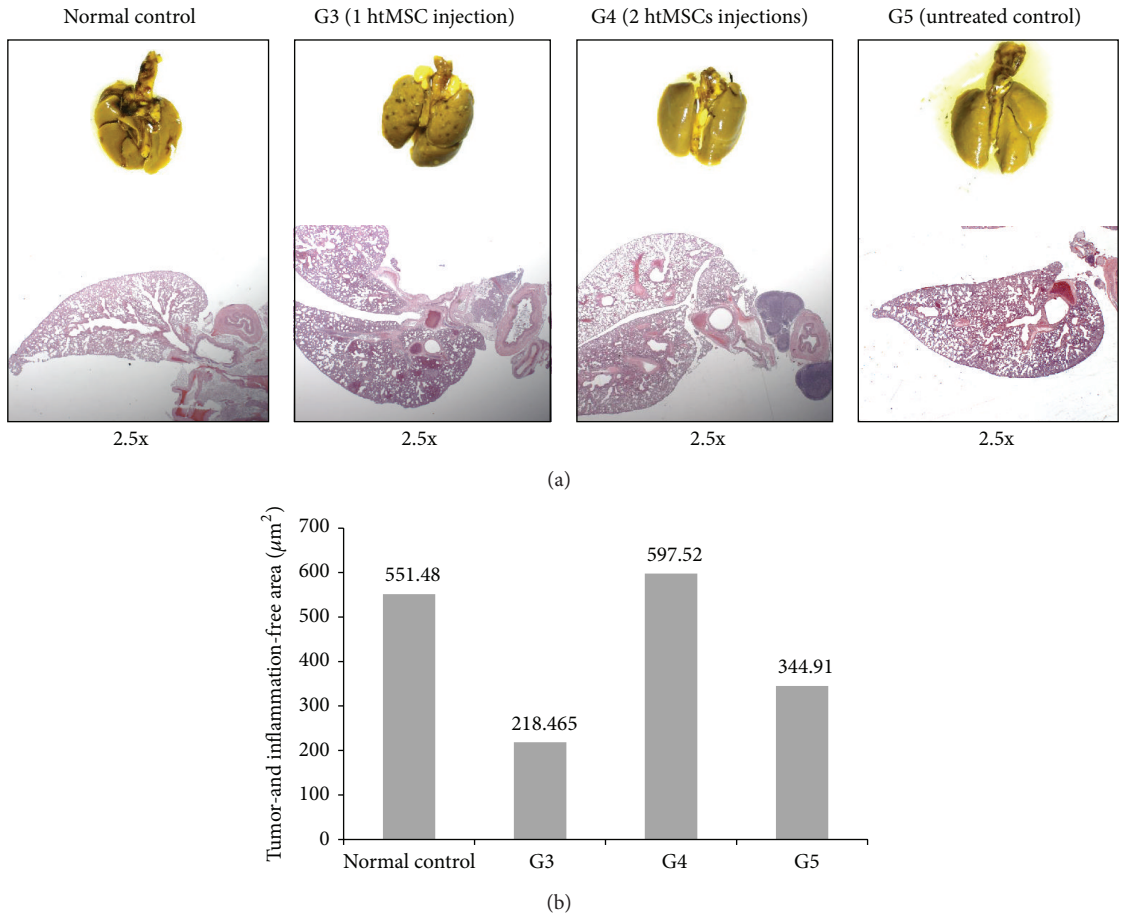


Figure 4: Inoculation of htMSCs in tumor-bearing mice. (a) Images show G3, G4, and G5 lungs (macroscopically and microscopically), in comparison with normal control (normal mice lung) 20 days after tumor cell inoculation. One representative animal of each group is shown. (b) Pulmonary tissue-free measurements, showing that lungs were preserved in G4 group (2 htMSCs injections), similar to normal controls, represented as tumor- and inflammation-free area.

there was a substantial increase (about 48% and 37%, resp.) in the secretion of IL-8 and MCP1. In contrast, a decrease in the levels of VEGF released by htMSCs (about 36%) was observed after cocultivation of cell lines in the described conditions. Due to the intraindividual variation of each htMSC analyzed, only the VEGF showed statistical significance, although a proinflammatory tendency is evident for other expressed cytokines (Il-6, IL-8, and MCP-1) (Figure 8).

4. Discussion

Here we show, for the first time, that human MSCs obtained from one single source and cultivated under the same conditions, when injected in animals with the same disease, can produce opposite results depending on the experimental protocol. When we compared the effect of subcutaneous coinjections of htMSCs and tumor cells with intraperitoneal injections of the same htMSC lineage in immunocompetent animals of the same age and background inoculated previously with the same tumor cells, we observed a beneficial effect only in animals in which the tumor was already established before the intraperitoneal htMSCs inoculation. When htMSCs were coinjected subcutaneously with 4T1 cells, we observed an opposite effect, that is, exacerbation on primary and metastatic tumor development.

Several mechanisms have been reported to be responsible for these discrepant observations, such as chemokine signaling, modulation of apoptosis, vascular support, and immune modulation. Suzuki et al. [21] showed that murine bone marrow MSCs increased local neovascularization and tumor growth. It has also been reported that human bone marrow MSCs increased tumor growth and metastasis in murine colon cancer [22].

On the other hand, it is well documented that MSCs release factors with angiogenic and immunomodulatory properties which was observed even in xenotransplantation of human MSCs in animal models [23, 24]. Therefore, in order to verify if the observed results could be related to cytokines and chemokines released by htMSCs in the tumor microenvironment, we performed *in vitro* cocultures of htMSCs and 4T1 cells.

After 48 hours of coculture with no direct contact of htMSCs and 4T1 tumor cells, we observed a significant increase of IL-8 (interleukin-8) and MCP-1 (monocyte chemoattractant

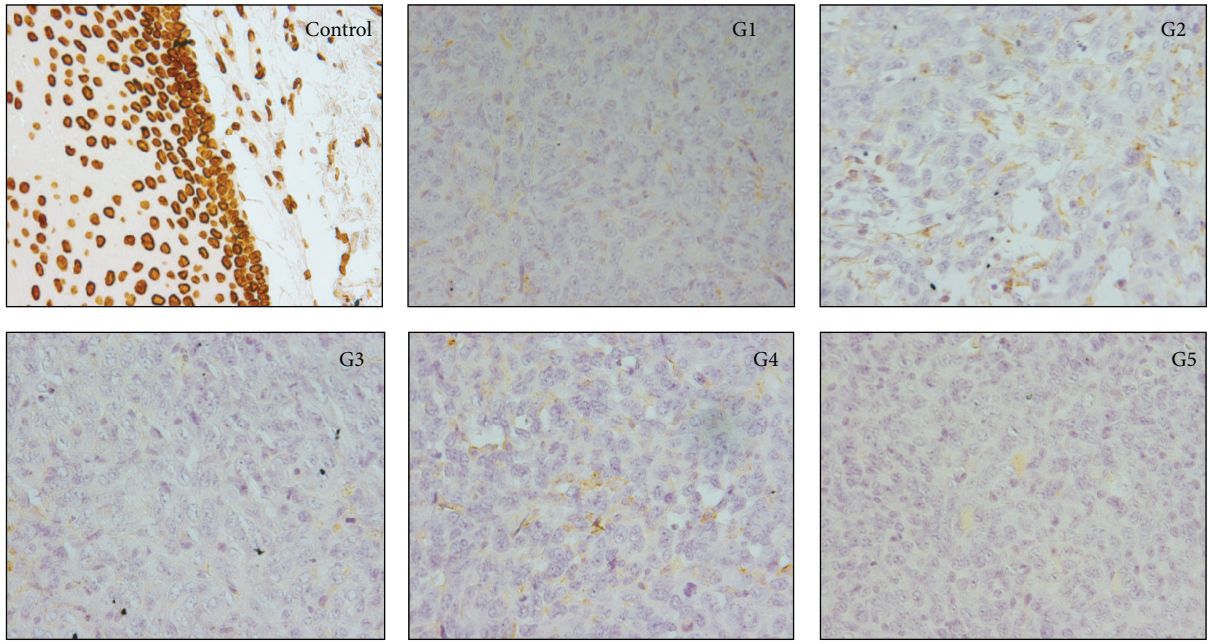


Figure 5: Human nuclei analysis in primary murine breast tumors, using the human specific antibody anti-lamin A + C, in G1 to G5 groups. Primary tumors were collected 15 days after tumor cell inoculation. Positive control (human tissue), showing positive staining. Slide of one representative animal of each group is shown. The antibody did not stain any structure in all primary tumors analyzed.

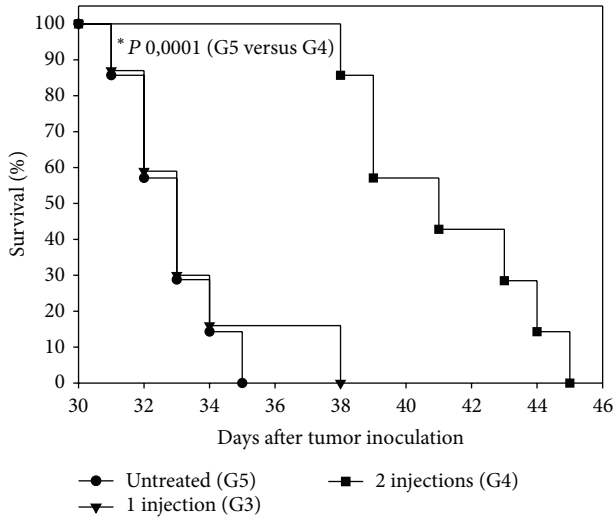


Figure 6: Survival analysis of tumor-bearing mice inoculated intraperitoneally with htMSCs. Animals (six animals per group) treated with 2 htMSCs injections (G4) showed a statistically significant increase in survival compared to untreated animals (control, G5).

protein-1), 44% and 37%, respectively, as well as a decrease (36%) of VEGF (vascular endothelial growth factor). This result shows that unknown factors released by murine tumor cells can regulate the production and secretion of IL-8, MCP-1, and VEGF by htMSCs.

IL-8, alternatively known as CXCL8, is a proinflammatory chemokine highly related to the progression of cancer, since many studies have shown overexpression of IL-8 by tumor cells. It is a chemotactic factor exerting a large migratory stimulus to immune system cells, especially neutrophils. It also determines an increase in the expression of adhesion molecules by endothelial cells [25]. Also, Fujimoto and colleagues [26] showed that MCP-1 induces tumor-associated macrophage infiltration and contributes to tumor progression in immunodeficient mice bearing human breast cancer cells by recruiting monocytes to injury sites, triggering thus a proinflammatory reaction. It has been shown that chemotactic proteins such as MCP-1 and IL-8 promote migration of human MSCs *in vitro* and induce the recruitment of leukocytes to the injured sites [27]. Therefore, the increased expression of IL-8 and MCP-1 we found in the coculture htMSCs/4T1 media suggests that the increased secretion of these molecules at the tumor microenvironment can be related to the increased tumor growth observed when these cells were coinjected *in vivo*. Although we were not able to detect human MSCs at established primary tumor sites, our results suggest that the interaction of these cells during the implantation period of tumor cells after coinjection can facilitate and stimulate tumor growth.

VEGF is a cytokine strongly related to angiogenesis regulated by microenvironmental factors within the tumors, such as hypoxia, free radicals, pH imbalance, and nutrient deficiency. Its expression may be influenced by a number of microenvironmental factors which may play important role in regulating VEGF expression during tumorigenesis [28]. On the contrary, proangiogenic factors can also have an immunosuppressive effect. Vascular endothelial growth factor A (VEGF-A) can induce the accumulation of immature dendritic cells, myeloid-derived suppressor cells, and regulatory T cells and inhibit the migration of T lymphocytes to the

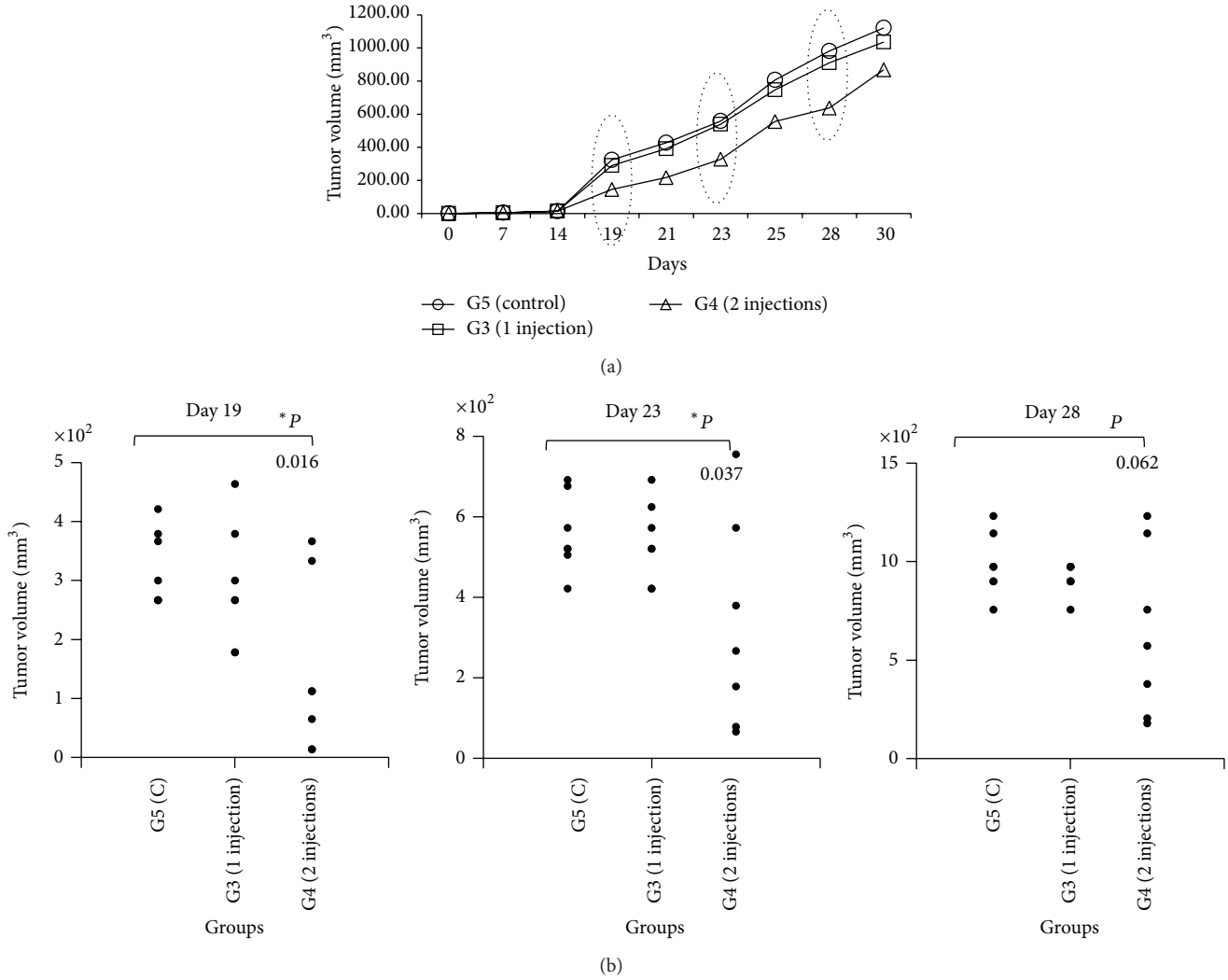


Figure 7: Primary tumor development in tumor-bearing mice inoculated intraperitoneally with htMSCs. (a) The average tumor volume of G3, G4, and G5 groups in each day is represented. (b) Tumor volumes of individual animals on days 19, 23, and 28. Animals injected with 2 doses of htMSCs showed significantly reduced tumor development compared to untreated control until 23 days after tumor inoculation.

tumor. It has been suggested that other proangiogenic factors such as placental growth factor (PlGF) could also participate in tumor-induced immunosuppression [29].

The reduced VEGF secretion by htMSCs in coculture with 4T1 cells suggests that the expression of IL-8 and MCP-1 by htMSCs at the tumor microenvironment after coinjection of mesenchymal and tumor cells strongly regulates the increased primary and metastatic tumor development, by chemoattracting secondary immune cells, with a minor participation of VEGF in these conditions. We hypothesize that the influence of these htMSCs-secreted factors at the beginning of tumor establishment at the mammary fat pad is very important for the exacerbation of tumor growth and metastasis.

Although our results showed that htMSCs were not found at the primary tumor 15 days after tumor cell inoculation, we cannot exclude that these cells were recruited to the tumor site immediately after intraperitoneal inoculation but could not survive long in this murine environment. The production of reduced concentrations of VEGF by htMSCs, leading to a less immunosuppressive tumor environment, in association with the recruitment of immune cells by IL-8 and MCP-1, after tumor cells establishment (first dose) and during tumor development (second dose), could explain the significant tumor growth control induced by the treatment protocol.

Corroborating our hypothesis that the immune system has an important participation in the effects observed after both protocols, it has been shown that another variant that could influence the role of MSCs in tumor development is the use of immunodeficient/immunosuppressed or immunocompetent animal models. According to Barcellos-de-Souza et al. [30], several *in vivo* assays that performed coinjections of MSCs with different types of tumor cells in immunocompromised animals showed an increase in tumor growth. Among them are models of colon cancer, osteosarcoma, ovarian cancer, colorectal cancer, melanoma, lung cancer,

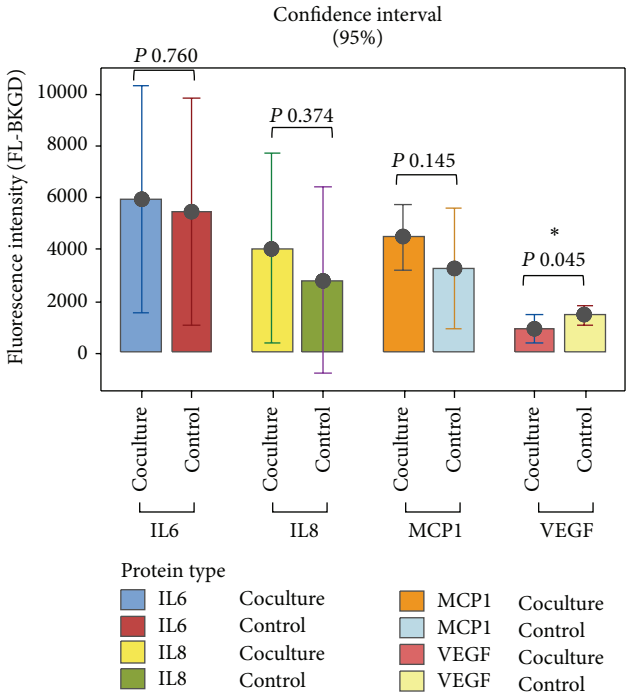


Figure 8: Concentration of cytokines released by htMSCs before (control) and after coculture with murine 4T1 tumor cells (with no direct contact). A small increase (about 9%) of IL6, an important increase of IL-8 and MCP1 (about 45% and 37%, resp.), and a decrease of VEGF expression (about 36%) were observed ($P = 0.045$). All samples were analyzed in triplicate. The results represent the mean and standard deviation of each triplicate.

gastric cancer, and prostate carcinomas. In opposition, Lu et al. [31] showed an inhibition of ascites formation in an immunocompetent murine model of ascitogenous hepatoma after three injections of murine bone marrow MSCs, zero, three, and ten days after tumor cell inoculation.

Previous studies from our and other groups have shown that MSCs from different sources, such as umbilical cord, dental pulp, and adipose tissue [32–34], may have different clinical effect when injected in animal models for neuromuscular disorders. However, some properties such as immunomodulatory potential are apparently a common characteristic of MSCs [35].

Here, we show that the same MSCs, injected in the same animal model, may lead to opposite results according to the experimental procedure. Our results reinforce that the moment when MSCs reach tumor microenvironment and apparently secrete factors to recruit other immune cells after interaction with tumor cells is crucial for tumor development.

We are not aware of other studies comparing the clinical effects of htMSCs in immunocompetent mice developing a breast adenocarcinoma. Therefore it is very important to verify whether the beneficial effect we observed in delaying tumor growth and increasing the life span of 4T1 breast tumor-bearing immunocompetent mice also occurs with MSCs from other sources. This is particularly relevant since any approach aiming to treat human cancer will be done in patients with established tumors.

5. Conclusions

In short, here we show that (1) htMSCs promote and/or accelerate breast adenocarcinoma in immunocompetent mice when coinjected with 4T1 tumor cells; (2) htMSCs can be beneficial to the animals that already have an established breast cancer at initial stages, depending on the dose and the route of administration of the injected htMSCs, decreasing primary and metastatic tumor growth and significantly increasing their survival; (3) repeating these experiments with MSCs from other sources is of utmost importance.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

E. G. Rodrigues and M. Zatz contributed equally to this work.

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Review Article

Endothelial Progenitor Cells in Tumor Angiogenesis: Another Brick in the Wall

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Until 15 years ago, vasculogenesis, the formation of new blood vessels from undifferentiated cells, was thought to occur only during embryonic development. The discovery of circulating cells that are able to promote vascular regeneration and repair—the so-called endothelial progenitor cells (EPCs)—changed that, and EPCs have since been studied extensively. It is already known that EPCs include many subtypes of cells that play a variety of roles in promoting vascular growth. Some EPCs are destined to differentiate into endothelial cells, whereas others are capable of promoting and sustaining angiogenesis through paracrine mechanisms. Vasculogenesis and angiogenesis might constitute complementary mechanisms for postnatal neovascularization, and EPCs could be at the core of this process. Although the formation of new blood vessels from preexisting vasculature plays a beneficial role in many physiological processes, such as wound healing, it also contributes to tumor growth and metastasis. However, many aspects of the role played by EPCs in tumor angiogenesis remain unclear. This review aims to address the main aspects of EPCs differentiation and certain characteristics of their main function, especially in tumor angiogenesis, as well as the potential clinical applications.

1. Introduction

In the past few years, a number of studies have shown that adult stem and progenitor cells play a role in tumor progression. Deregulation in the self-renewal programs of adult stem cells leads to cell transformation, contributing to the formation and development of new tumors [1]. Although angiogenesis (the formation of new blood vessels from preexisting vasculature) plays a beneficial role in many physiological processes, such as wound healing, it also contributes to the growth and metastasis of tumors.

Until the 1990s, postnatal neovascularization was thought to result from the detachment and proliferation of mature endothelial cells, supporting the idea that vasculogenesis (the formation of new blood vessels from progenitor cells or angioblasts) occurs only during embryogenesis. In 1997, Asahara et al. [2] isolated mononuclear cells from adult peripheral blood and found that those cells had the same characteristics as the embryonic angioblasts that contribute to the revascularization of ischemic tissue. In a subsequent study, Asahara et al. [3] coined the term "endothelial progenitor cells" (EPCs) to describe these cells. In that study, the authors showed that bone marrow-derived EPCs not only have therapeutic applications but also are involved in the pathological neovascularization of tumors and consequently in their growth. In 2004, Asahara and Kawamoto [4] proposed that vasculogenesis and angiogenesis constitute complementary mechanisms of postnatal neovascularization in which EPCs can play a role. More recently, studies have indicated that adult progenitor cells have the ability to migrate and proliferate, contributing to the *de novo* formation of capillary structures [5]. Therefore, EPCs have been defined

as circulating progenitor cells that have the ability to differentiate and form functional blood vessels. However, the exact origin, character, and function of EPCs are still controversial in the literature, and their role in tumorigenesis is therefore also still under discussion. Here, we present the main issues involved in the characterization of EPCs and their role in angiogenesis, mainly in the promotion of tumor progression.

2. Characterization of EPCs

Human $CD34^+$ cells isolated from circulating peripheral blood, umbilical cord blood, or bone marrow can differentiate into endothelial cells [2, 6], as well as being capable of contributing to neoendothelialization and neovascularization in the adult organism. These cells can promote angiogenesis by two different mechanisms [7–10]: serving as the substrate for new vessel formation and exerting a paracrine effect. In fact, there are two main cell types within the EPC designation [11–16]: early EPCs (angiogenic cells), which have features of hematopoietic cells, can generate monocytic cells, and play a role in vasculogenesis by secreting large quantities of angiogenic factors that act via paracrine mechanisms, and late EPCs (endothelial outgrowth cells), which are able to differentiate into endothelial cells and promote vascular tube formation.

Although the functions of EPCs have been well described, their defining characteristics remain controversial in the literature. In general, EPCs have the ability to absorb acetylated low-density lipoprotein and to bind the lectin *Ulex europaeus* agglutinin I. Endothelial outgrowth cells differ from angiogenic cells due to their higher proliferative potential and their ability to promote the formation of vascular structures [12–16]. It is well known that, during hematoendothelial development, $CD34^+$ cells do not express CD45, rather acquiring it during differentiation into hematopoietic progenitor cells, except if they are destined to differentiate into endothelial cells [7, 17–19]. Moreover, CD34 antigen has its expression gradually reduced as the level of maturation of hematopoietic cell lineages increases [20]. Therefore, CD45 and CD14 are mainstream antigens able to differentiate these cell types (Table 1), because endothelial outgrowth cells originate from $CD34^+$ cells that are negative for CD45 and CD14, whereas angiogenic cells are $CD45^+/CD14^+/CD34^{low}$ cells (Figure 1).

Classically, surface immunophenotyping of EPCs was expected to express CD34, vascular endothelial growth factor receptor-2 (VEGFR-2), and prominin 1 (CD133) [4]. However, some studies have suggested that $CD34^+/VEGFR\text{-}2^+/CD133^+$ cells constitute an enriched population of $CD45^+$ hematopoietic precursors, or even mature circulating endothelial cells, and therefore do not contribute to the formation of endothelial cells *in vitro* [7, 21, 22]. In addition, stem/progenitor cells of other origins are also capable of differentiating into endothelial cells and exist either in the bone marrow—including $CD34^-/CD133^+$ multipotent adult progenitor cells [23, 24], mesodermal progenitor cells [25], and side population cells [26]—or in the peripheral blood—including circulating endothelial precursors that can be derived from stem/progenitor cells in bone marrow or can arise by detachment of mature endothelial cells or perivascular cells, such as pericytes [27]. Furthermore, EPCs can express certain endothelial markers, such as platelet-endothelial cell adhesion molecule-1 (CD31), Cdh5 (vascular endothelial cadherin), and von Willebrand factor (Figure 1) [28, 29].

Because the characterization of EPCs is controversial, additional criteria for defining EPCs, based on morphology and culture procedures, have been established (Table 1). To isolate and expand EPCs from umbilical cord and peripheral blood mononuclear cells, three culture methods have been described [31]. The first involves culturing mononuclear cells on fibronectin-coated dishes and replating the nonadherent cells after 48 h. In that method, angiogenic cells arise after 4–9 days as round cells surrounded by spindle-shaped cells. The second method involves culturing mononuclear cells on fibronectin-coated dishes, in this case for 4 days, and keeping the adherent cells, which give rise to a heterogeneous population of cells termed circulating angiogenic cells. These cells do not form colonies or express endothelial cell surface antigens but do retain the characteristics of monocytes. The exact origin and role of these cells *in vivo* are a question that remains unanswered, and they may come from detachment of endothelium layer cells, mesenchymal stem cells, or hematopoietic stem cells [27]. The third culture method involves a longer period of mononuclear cell culture on collagen-coated dishes. Between days 7 and 21, a population of cells originates from the adherent cells, and that population has been characterized as being composed of endothelial outgrowth cells [32, 33]. Colonies of endothelial outgrowth cells are identified by their cobblestone-like structure [14, 34] and are cultivated in medium supplemented with growth factors such as epidermal growth factor, VEGF, basic fibroblast growth factor (bFGF), insulin-like growth factor-1, ascorbic acid, and a gentamicin-amphotericin B mix. However, it has been shown that the presence of mesenchymal stem cells induces EPCs to differentiate into endothelial cells, promoting angiogenesis even without the addition of exogenous growth factors [35], indicating the important roles that paracrine effects and direct cell contact of these cell subtypes play in the modulation of the angiogenic response. Bone marrow-derived EPCs have also been isolated from mononuclear cell phase or whole bone marrow cell extract and cultivated in fibronectin-coated dishes for 7 days. After two days of culture, a small "blood island" appears, and cells adhere to the plate, exhibiting spindle-like appearance, after seven days [36].

It seems that the differentiation of endothelial cells and their maturation as vascular cells, together with the formation and stabilization of new blood vessels, constitute a dynamic and complex process that comprises different types of cells, each of which has a specific function and all of which are essential to the final result. Although the phenotype and characterization of EPCs have yet to be fully determined, their origins and functions have been well established in the last decade [2–5, 21–27]. The challenge now is to understand the mechanisms involved in the formation of new blood vessels and how they lead to benefit or harm.

Table 1: Main characteristics distinguishing endothelial outgrowth cells and angiogenic cells.

Characteristic	Endothelial outgrowth cells	Angiogenic cells
Time for culture growth	7–21 days	3–5 days
Morphology	Confluent cobblestone monolayer	Round to spindle-shaped
Surface antigens	$CD34^+$ VEGFR-2^+ $CD45^-$ $CD14^-$ $CD133^-$	$CD34^{low}$ VEGFR-2^{low} $CD45^+$ $CD14^+$
Nitric oxide production	High	Low
Cytokine secretion	Low	High
Vascular tube formation	Generate vascular tubes in Matrigel	Do not generate vascular tubes in Matrigel
Neovascularization capacity	Improve neovascularization	Improve neovascularization
Cell properties	Bind UEA-I lectin and take up LDL	Bind UEA-I lectin and take up LDL

VEGFR: vascular endothelial growth factor receptor; UEA-I: *Ulex europaeus* agglutinin I; LDL: low-density lipoprotein.

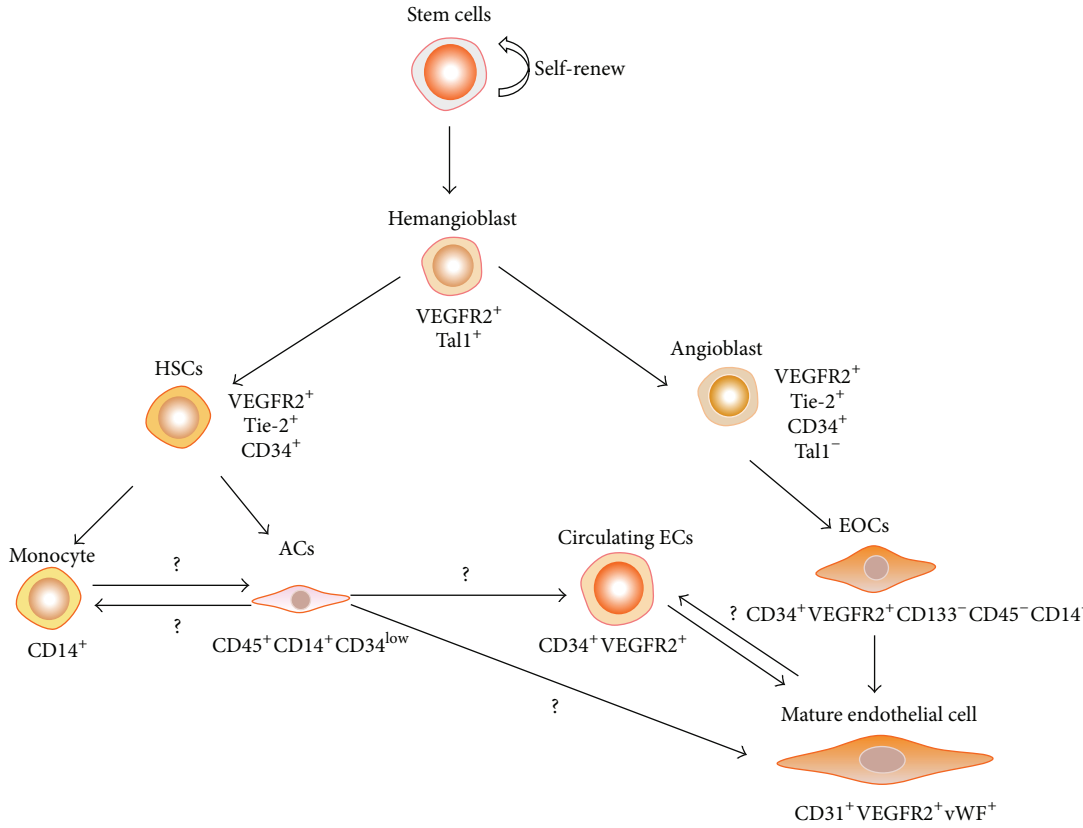

Figure 1: Adult endothelial progenitor cell phenotype (ACs: angiogenic cells; ECs: endothelial cells; EOCs: endothelial outgrowth cells; HSCs: hematopoietic stem cells; VEGFR: vascular endothelial growth factor receptor). Hemangioblasts derived from pluripotent stem cell can differentiate into HSCs and angioblasts. The HSCs give rise to blood cells, such as monocytes and ACs. Whether monocytes can act as ACs and vice versa is still controversial. Angioblasts give rise to endothelial cell lineage, including EOCs. Circulating endothelial cells can arise from the detachment of mature ECs and repair other areas of endothelium damage or can arise from the differentiation of ACs.

3. Molecular Signaling in Angiogenesis

Vasculogenesis is defined as the formation of the primitive vasculature network during the embryonic period, whereas angiogenesis is defined as the formation of new blood vessels from preexisting vasculature. However, because adult EPCs have now been identified, the best term to describe this complex process in which mature and progenitor endothelial cells take part would be postnatal vasculogenesis. Although the formation of new blood vessels is vital to many beneficial physiological processes, such as wound healing and bone repair [37, 38], it can also be involved in pathological conditions, including arthritis [39] and diabetic retinopathy [40], as well as tumor growth and metastasis [41]. Therefore, angiogenesis is largely studied as a target of new therapeutic strategies.

Angiogenesis and its role in tumor growth were first described in the 1970s by Ausprunk and Folkman [42]. It is now known that angiogenesis involves two separate processes. The process described by Ausprunk and Folkman [42], designated sprouting angiogenesis, is characterized by migration, proliferation, three-dimensional organization, and tube formation of endothelial cells. More recently, another process, known as nonsprouting angiogenesis (intussusception), has been described and is defined as the division of vessels by transluminal pillar formation through invagination with interstitial tissue [43].

The induction of angiogenesis relies on a tenuous balance between pro- and antiangiogenic factors. The proangiogenic factors include bFGF [44], platelet-derived growth factor [45], platelet-derived endothelial cell growth factor [46], angiopoietin-1 [47], transforming growth factor beta-1 [48], transforming growth factor alpha, and epidermal growth factor [49]. However, the most well-known proangiogenic factor is VEGF-A. Other VEGF family subtypes, such as VEGF-B, VEGF-C, VEGF-D, and placental growth factor, have also been shown to be involved in tissue-specific forms of angiogenesis, including myocardial angiogenesis [41], embryonic angiogenesis, and lymphangiogenesis. In addition, VEGF-A stimulates mitogenesis and cell migration, as well as increasing vasodilatation and vascular permeability. These effects are mediated by activation of tyrosine kinase receptors (VEGFRs), which are present on the cell surface.

It has been demonstrated that VEGF-A binds to VEGFR-1 and VEGFR-2 [50]. Most of the biological effects of VEGF-A are mediated by VEGFR-2, whereas VEGFR-1 activation is still not completely understood. Despite the fact that VEGF-A binding to VEGFR-1 is 10-fold higher than VEGF-A binding to VEGFR-2 [51], some evidence suggests that VEGFR-1 mediates angiogenesis during embryogenesis [52], whereas the architectural organization of new blood vessels without mitogenic activity is controlled by VEGFR-2 [53]. In addition, because of the high VEGFR-1 binding affinity without activation of downstream signaling, VEGFR-1 is considered to be a trap receptor, making VEGF-A less available to VEGFR-2 binding [54]. Other receptors for different isoforms of VEGF were also described. Neuropilin-1 and neuropilin-2 were originally identified as receptor for axon guidance factors belonging to semaphorins family. Subsequently, the binding of VEGF isoforms to these receptors revealed their participation in the angiogenesis modulation [55]. VEGF165 isoform binds to neuropilin-1 and increases the proliferation and migration of cells that express VEGFR2 [56], suggesting that neuropilins may interact with VEGFRs contributing to physiological and pathological angiogenesis [57]. In addition, semaphorins have also been shown to favor tumor growth by promoting angiogenesis [58].

Activation of VEGFR-2 induces various intracellular signaling pathways. After VEGF-VEGFR-2 binding, tyrosine phosphorylation activates phospholipase C, thus increasing inositol triphosphate levels, which leads to Ca^{2+} fluxes. This process also generates diacylglycerol, thereby activating protein kinase C and inducing activity of extracellular-signal regulated kinases (ERK) 1 and 2, resulting in proliferation [50]. In addition, VEGFR-2 binds to phosphoinositide 3-kinase, which has been implicated in the tube formation, proliferation, survival, and vascular permeability of endothelial cells [59]. These are the signals that contribute to angiogenesis promotion, and the inhibition of this pathway has been the object of numerous studies aimed at increasing the efficacy of antiangiogenic treatment strategies in patients with tumors. Although most such studies have focused on therapies that block VEGF signaling, there are other factors that regulate the VEGF pathways.

Various products of the alternative splicing of VEGF-A mRNA have antiangiogenic properties. A shift in the balance of alternative splicing (toward expression of pro- or antiangiogenic VEGF isoforms) is modulated by physiological or pathophysiological processes [60]. Other molecules, such as semaphorin 3E, which binds to the neuropilin receptor, are also involved in the inhibition of angiogenesis [61]. Therefore, in addition to understanding how to inhibit angiogenic factors, it is thought to be important to establish an antiangiogenic balance in order to create the conditions that would allow new therapeutic strategies to inhibit or stimulate angiogenesis.

Hypoxia (the loss of vascular function leading to low oxygen tension) is the main trigger for the elaborate process of angiogenesis. Damaged tissue and tumor tissue both present hypoxic environments. Various mechanisms are triggered when there is a need to restore the supply of oxygen. The most important is the activation of transcription factor hypoxia-inducible factor-1 (HIF-1), which induces the expression of adhesion molecules [62], matrix components [63, 64], metabolic proteins [65], and growth factors, such as VEGF-A [66, 67]. Thus, physiological and pathological angiogenesis both result from an imbalance between pro- and antiangiogenic factors, favoring the former.

Once VEGF-A is expressed, it becomes the major player in angiogenesis. Produced by a variety of different cell types, such as macrophages, platelets, retinal epithelial cells, tumor cells, and endothelial cells, VEGF-A has antiapoptotic effects and is a potent mitogen. It also stimulates the production of adhesion molecules [62], matrix components [63, 64], and matrix metalloproteinases (MMPs) [65]. The main targets of VEGF-A are endothelial cells, where it stimulates the production and release of nitric oxide (NO), which causes local vasodilatation. In addition, after the release of nitric oxide, endothelial cells change their shape and cell-to-cell adherence is reduced, resulting in increased vascular permeability, which allows circulating cells and proteins to reach the site of injury. Nevertheless, some tissues have no capacity to regenerate after an injury because they are avascular. It has been demonstrated that the use of VEGF-A in the treatment of tears in the medial menisci, whose lateral two-thirds are avascular, does not promote tissue repair because there is no formation of a complex vascular bed [66]. These data suggest that angiogenesis relies on a network of complex events rather than VEGF-A stimulation alone. Furthermore, most avascular tissues have an intrinsic mechanism that blocks this process. Therefore, angiogenesis not only requires a shift from an antiangiogenic to a proangiogenic balance but also

depends on other concurrent events that do not always take place.

Unlike the vascularization that occurs during tissue repair, the vascularization of tumors is characterized by a chaotic network composed by vessels of different calibers and often with blind ends. One explanation for this characteristic is that HIF-1 expression promotes the constant release of bFGF and VEGF-A, recruiting cells to the site of vessel formation in an unregulated manner [67].

4. EPCs in Cancer Angiogenesis

Although the molecular mechanisms involved in the activation of angiogenesis are well understood, many antiangiogenic therapies have failed due to alternative molecular escape routes [68]. In this context, another important point to be considered is the cellular component of the angiogenesis process. Until the last decade, it was thought that angiogenesis was promoted only by stimulation of mature endothelial cells. Recent studies have demonstrated the role played by EPCs. Ischemic stimuli are often sufficient to recruit EPCs from bone marrow [2, 8, 24, 69], after which they can incorporate into sites of injury and promote vascularization. Additional evidence of EPC-related protection in vascular diseases comes from the finding that there is an inverse correlation between the number and quality of circulating EPCs in the peripheral blood of patients with cardiovascular impairment [7, 70–74].

It has been demonstrated that EPCs migrate to sites of vascular damage, such as an ischemic limb, an injured retina, and an infarcted myocardium, where they increase vascularization, as well as improving blood flow and tissue performance [8, 26, 69, 75]. Studies of treatment with mature endothelial cells and EPCs have shown that the latter induce neovascularization after myocardial infarction [8, 76], suggesting that EPCs reduce ischemic damage by promoting angiogenesis and that mature endothelial cells do not.

Despite the knowledge that EPCs are a feasible treatment for a variety of clinical conditions, it remains unclear which specific type of EPC would be most appropriate for use in stem cell therapy. The great majority of the studies on the topic have failed to determine whether the injected cells were angiogenic cells or endothelial outgrowth cells, and the initial characterization of the cells has often been incomplete. Although the injection of only one type of EPC induces neovascularization [14, 77], treatment with both types promotes significantly greater tissue repair [14]. Therefore, bidirectional communication between angiogenic cells and endothelial outgrowth cells appears to be important for improving physiological angiogenesis. However, the chaotic tumor microenvironment leads to dysfunction of angiogenic stimuli and formation of a disorganized vascular network. Many cancers, such as some forms of leukemia, lymphoma, and breast cancer [78], have been associated with an increase in the number of circulating EPCs. In addition, EPC recruitment favors tumorigenesis. In a study involving xenotransplantation of human tumors in mice, the size of the tumors was found to increase when EPCs were injected systemically, which resulted in better vascular network formation within the tumor microenvironment [28].

Tumors produce growth factors that modulate angiogenesis, including VEGF-A and bFGF. The release of such growth factors and the hypoxic tumor microenvironment recruits EPCs from bone marrow or activates tumor residents EPCs. Those EPCs either differentiate into endothelial cells or produce angiogenic growth factors. In brief, tumor hypoxia favors the rupture of the extracellular matrix (ECM) due to the release of MMPs, which contribute to tumor angiogenesis and metastasis. In addition, within the tumor microenvironment, mesenchymal stem cells and other cells, including pericytes, can constitute an additional source of proangiogenic factors, playing an important role in covering and protecting newly formed vessels. The release of VEGF-A not only induces tumor angiogenesis but also inhibits the recognition and destruction of tumor cells by the immune system [79]. Neovascularization ensures an adequate supply of oxygen and blood for tumor progression, as well as facilitating metastasis, because it provides a route by which tumor cells get into the bloodstream and spread throughout the system, an increase in vascular density having been shown to increase metastatic potential [80].

The role of EPCs in promoting tumor angiogenesis and metastasis has been the target of many studies aimed at developing new therapeutic strategies. However, identifying the origin of these cells and determining their exact location after injection continue to pose challenges. Some authors believe that hematopoietic stem cells, mesenchymal stem cells, and EPCs reside in the stroma surrounding the tumor mass. Melero-Martin and Dudley [30] showed that paracrine crosstalk between tumor cells and stromal cells results in an unexpected pattern of stem/progenitor cell differentiation, which could accelerate the progression of the tumor. The authors argued that whereas EPCs are the primary agents of lumen formation in new angiogenic sprouts, mesenchymal stem cells and hematopoietic stem cells act as angiogenic stimulators by secreting VEGF-A, as well as tissue remodeling and endothelial survival factors that sustain the process of angiogenesis. Mesenchymal stem cells can also differentiate into pericytes, which support the formation of new blood vessels. Therefore, these three populations of stem/progenitor cells work in concert to form the building blocks of tumor vascularization (Figure 2).

The overarching question is how the activation of stem/progenitor cells present in the stroma of a tumor is coordinated. One hypothesis is that ECM remodeling around the tumor mass can provide signals to the stem/progenitor cells regarding their differentiation and plasticity [81]. Because ECM compounds are important to EPC growth and differentiation [82, 83], disruption of the ECM by tumor cells can lead to activation of the "incorrect" EPC fate. A second clue to the deregulation of the fate of stem/progenitor cells is epigenetic alterations caused by their interaction with the tumor microenvironment. Extracellular factors released by tumor cells regulate epigenetic alterations in cells within the stroma of the tumor, promoting its growth and metastasis. In a study of prostate cancer, changes in the epigenetic patterns of endothelial cells in the tumor microenvironment showed

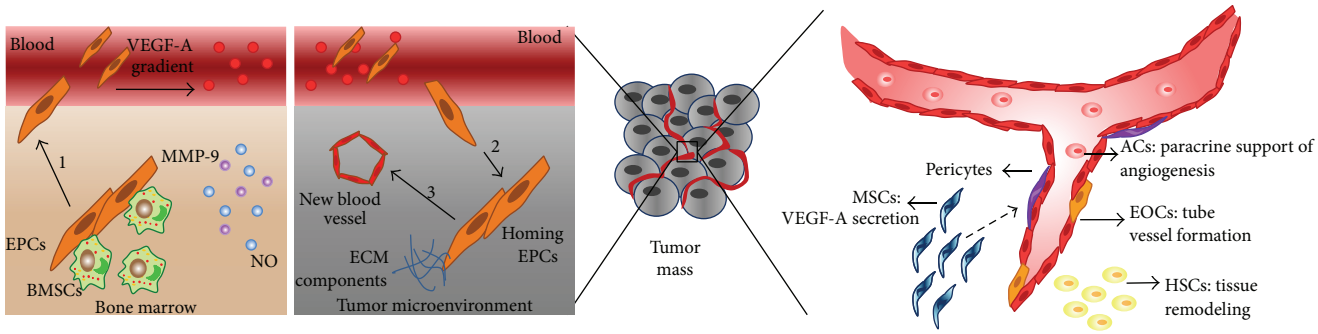

FIGURE 2: EPCs are the major players in new vessel formation contributing to tumor growth and metastasis. They might be recruited from bone marrow and migrate to the tumor (on the left) or either reside within tumor stroma, where there are other stem/progenitor cells that promote and/or contribute to new vessel formation (on the right: ACs: angiogenic cells; BMSCs: bone marrow-derived stem cells; ECM: extracellular matrix; EOCs: endothelial outgrowth cells; EPCs: endothelial progenitor cells; HSCs: hematopoietic stem cells; MMP: metalloproteinase; MSCs: mesenchymal stem cells; NO: nitric oxide; VEGF: vascular endothelial growth factor). Figure adapted from Melero-Martin and Dudley [30] (license number 3593771424850).

that methylation of the promoter of the gene *CYP24A1* plays a role in determining the phenotype of the tumor-associated vasculature [84]. In addition, hypermethylation of a specific tumor suppressor gene in mesenchymal stem cells has been shown to cause those cells to display various features of cancer stem-like cells/cancer-initiating cells, including loss of anchorage dependence, increased colony formation capability, drug resistance, and pluripotency [85]. These stromal cells might coevolve with tumor cells during tumor progression, acquiring the characteristics of nearby tumor cells, thus contributing to the formation of new blood vessels.

It seems that VEGF-A mediates not only tumor angiogenesis but also the maintenance of the stem cells surrounding the tumor. Beck et al. [86] showed that blocking VEGFR-2 results in tumor regression because it decreases microvascular density and reduces the size of the stem cell pool, thereby impairing their renewal capacity. Those authors identified a dual role for tumor cell-derived VEGF-A in promoting cancer stemness: by stimulating angiogenesis in a paracrine manner, thus creating a perivascular niche for stem cells, and by directly affecting stem cells in an autocrine loop, thus stimulating cancer stemness and renewal.

Unlikely, Melero-Martin and Dudley [30] and Nolan et al. [87] proposed a different origin for cancer EPCs. They demonstrated that bone marrow-derived EPCs are the main source of endothelial cells that could contribute to neovascularization, mainly in early tumors [86]. In addition to vascular tube formation in tumors [88, 89], bone marrow-derived EPCs have also been shown to participate in a paracrine fashion by exerting self-enhancement effects and regulating the expression of interleukin-1 beta in THP-1 monocytes [90], as well as that of monocyte chemotactic protein-1 in hepatocellular carcinoma [91]. Therefore, the success of tumor angiogenesis promoted by bone marrow-derived EPCs depends on the occurrence of three consecutive events: recruitment of the EPCs from the bone marrow to the peripheral blood, EPC homing to and invasion of the tumor site, and EPC differentiation into mature endothelial cells for the formation of new blood vessels (Figure 2). The recruitment of EPCs from bone marrow is regulated by a variety of growth factors, enzymes, ligands, and membrane receptors. The most important factor in the recruitment of EPCs is VEGF-A, which, upon stimulation by a protease, typically MMP-9 induced by endothelial nitric oxide synthase activity, allows the detachment of EPCs and their release into the systemic circulation. The release of EPCs is regulated by stromal cell-derived factor-1, also known as chemokine (CXC motif) ligand 12 (CXCL12), and its receptor, chemokine (CXC motif) receptor 4 (CXCR4), as well as by bone marrow integrins [92–94].

The migration of EPCs to the site of a tumor occurs by chemokine gradients that activate their correspondent cell receptors. The main participants are VEGF-A/VEGFR-2, CXCL12/CXCR4, growth-regulated oncogene alpha/CXCR1, interleukin-8/CXCR2, chemokine (C-C motif) ligand 2 (CCL2)/chemokine (C-C motif) receptor 2 (CCR2), and CCL5/CCR5 [78], and it seems that they act mutually, because CXCL12 expression depends on the quantity of VEGF-A [95, 96]. Once in the tumor bed, EPCs interact with endothelial cells via selectins, integrins, and adhesion molecules, allowing adhesion and migration to the site where new vascularization is needed [78]. After transendothelial migration and tissue homing, EPCs interact with ECM compounds to induce cell differentiation. Fibronectin is the major factor promoting VEGF-induced differentiation of EPCs in mature endothelial cells [97]. Therefore, crosstalk among endothelial cells, pericytes, and hematopoietic stem cells favors ECM remodeling to support the maturation and stabilization of the network of capillary tubes [98].

In general, there are two schools of thought regarding tumor angiogenesis promoted by EPC activation: one that promotes the idea that stem/progenitor cells reside in the tumor microenvironment and that certain factors disrupt their fate and stemness and the other that subscribes to the idea that EPCs are recruited from the bone marrow to the tumor site. Based on their intrinsic ability to home to tumor sites, EPCs are attractive as cell vectors for targeted cancer gene therapy. Scientists have developed genetically

engineered EPCs, transfected with vectors encoding some specific antitumor molecules. In that approach, the cells retain their homing properties but lose their capacity to form new blood vessels. In animal models of melanoma, delivery of specific MMP-12 by such EPCs has been shown to induce cleavage of molecules that induce tumor progression, thus inhibiting tumor growth, angiogenesis, and metastasis [99]. The same type of therapy has also prolonged the survival in tumor-bearing mice. The genetically modified EPCs release CD40 ligand that induces the production of tumor necrosis factor and interferon gamma, as well as increasing the activity of caspase-3 and caspase-7, in metastatic lung cancer [100]. In addition, it has been demonstrated that these cells enhance antitumor effects by inhibiting angiogenesis and inducing apoptosis in a murine model of glioma [101].

5. Conclusion

In the promotion of angiogenesis, EPCs are crucial, and they are attracted to hypoxic environments. Characterizing the various types of EPCs is hard work, and there have been some studies aimed at clarifying the distinctions among them. Some cell markers, such as CD45 and CD14, might be useful in distinguishing endothelial outgrowth cells, which differentiate into mature endothelial cells, being the real building blocks of the vessel walls, from angiogenic cells, which are derived from hematopoietic stem cells producing diverse cytokines that initiate and maintain the angiogenesis cascade.

Angiogenic cells and endothelial outgrowth cells have both been used in the treatment of ischemic diseases, either in animal studies or in clinical trials, and favorable results have been obtained. When the two cell types have been used in combination, therapeutic goals have been met in a more satisfactory manner, indicating that they act in concert. Despite the beneficial effect of EPC-induced angiogenesis during wound healing, such angiogenesis is actually harmful when it occurs in a tumor, contributing to its growth and metastasis. However, because of ability of EPCs to migrate and home to a tumor site, their use as a vector in gene therapies has become a promising therapeutic strategy. In this review, the main role of EPCs in the angiogenesis process was described. A better understanding of the molecular biology of EPCs could facilitate the identification of new targets for cancer therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Combined Effects of Pericytes in the Tumor Microenvironment

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Pericytes are multipotent perivascular cells whose involvement in vasculature development is well established. Evidences in the literature also suggest that pericytes display immune properties and that these cells may serve as an *in vivo* reservoir of stem cells, contributing to the regeneration of diverse tissues. Pericytes are also capable of tumor homing and are important cellular components of the tumor microenvironment (TME). In this review, we highlight the contribution of pericytes to some classical hallmarks of cancer, namely, tumor angiogenesis, growth, metastasis, and evasion of immune destruction, and discuss how collectively these hallmarks could be tackled by therapies targeting pericytes, providing a rationale for cancer drugs aiming at the TME.

1. Introduction

It has become increasingly evident that, not only the evolving genetic aberrations in malignant cells are critical in the pathophysiology of cancer, but also the interaction among cancer cells, nonmalignant cells, soluble factors, and other elements of the tumor microenvironment (TME). In addition to cancer associated-fibroblasts, immune cells, and endothelial cells (ECs), pericytes are also one of the main cellular components of the TME, whose diverse functions in tumor initiation and progression have only been recently addressed [1].

Pericytes were first described in the 19th century, at that time named "adventitial cells" by Rouget [2]. The term "pericyte" would only be applied in 1923, by Zimmermann [3]. These cells are commonly located on microvessel walls, within the basement membrane and closely opposed to the endothelium.

Under the microscope, pericytes are typically described as highly elongated, slender, and branched cells, with projections that extend longitudinally and circumferentially around the vessel wall [4, 5]. Pericytes have also been characterized by the expression of alpha-smooth muscle actin (α-SMA), desmin, CD146, platelet-derived growth factor beta receptor (PDGFRβ), and nerve/glial antigen-2 (NG2) proteoglycan [5, 6]. These markers, however, are not exclusive of pericytes and their expression may also vary according to the type of tissue, maturation stage, and pathological conditions [5, 7]. The use of different markers or combination of markers varies in the literature and, so far, a consensus about the phenotypic identity of pericytes has not been reached. Nonetheless this issue needs to be considered to better understand pericyte biology.

For instance, in a study using double transgenic Nestin-GFP/NG2-DsRed mice, Birbrair et al. [8] identified two pericyte subpopulations from large blood vessels and small capillaries, named type-1 and type-2 pericytes. These cell subpopulations expressed common pericyte markers, such as PDGFRβ, CD146, and NG2, but differed in Nestin expression. These distinct pericyte subtypes were later functionally characterized and shown to differ in their multipotent properties [8, 9] and angiogenic potential [10]. *In vitro* and *in vivo* assays revealed that type-2 (Nestin-GFP+/NG2-DsRed+), but not type-1 (Nestin-GFP−/NG2-DsRed+), pericytes are recruited during tumor angiogenesis. However, little is known about the ontogeny of these distinct subtypes and whether they are interconvertible.

In fact, the essential contribution of pericytes to vasculature development and maintenance has long been known. They participate in the regulation of blood flow and vessel permeability, as well as in stabilization of the vascular

wall [11]. Pericytes also provide important mechanical and physiological support to ECs and such interaction is essential for vessel remodeling and maturation [12, 13]. More recently, there have been growing evidences supporting new roles for pericytes in immunomodulation [14] and adult stem cell biology [15].

In the context of cancer, these distinctive pericyte properties make them important modifiers of disease progression, contributing directly or indirectly to tumor growth, metastatic spread, and resistance to therapy.

2. Tumor Angiogenesis

Tumor-driven angiogenesis was first described more than 100 years ago [16]. The later observation that without an efficient blood supply tumors could not grow beyond a critical size or metastasize stimulated an intensive search for pro- and antiangiogenic molecules [17, 18]. Nowadays, some of the latest therapeutic options for treatment of different cancers rely on antiangiogenic strategies, such as bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF).

Angiogenesis is a multiple-step process tightly orchestrated by many molecules that regulate both ECs and pericytes activities. Pericytes are known to secrete growth factors that stimulate EC proliferation, in addition to proteases that contribute to modulate the surrounding extracellular matrix and guide EC migration [13, 19–21]. The proliferative endothelium from a preexisting structure with the basement membrane forms an initial tube still in an immature state. Subsequently, ECs release signals that induce pericyte recruitment [22]. The resulting pericyte coverage is crucial for vessel remodeling, maturation, and stabilization.

The reciprocal communication between ECs and pericytes is established by direct contact, by paracrine signaling, or by a newly described chemomechanical signaling pathway [23]. Some of the signaling molecules involved in this crosstalk coordination include angiopoietin-1/2 and Tie2 (Ang/Tie2), transforming growth factor-β (TGF-β), and platelet-derived growth factor-β (PDGFβ/PDGFR-β), which are mainly related to EC viability, mural cell differentiation, and pericyte recruitment, respectively [24].

Similar events occur during tumor angiogenesis. The sprouting of ECs is followed by a pericyte migration but, in this case, the vascular architecture does not accomplish complete maturation, which leads to several structural and functional abnormalities [25, 26]. Tumor vessels are highly disorganized, irregularly shaped, tortuous, excessively branched, and leaky [27]. The basement membrane is discontinuous or absent and presents altered composition [28]. The endothelium can be incomplete or occasionally multilayered. ECs and perivascular cells also differ functionally and morphologically from their normal counterparts [29, 30]. In tumor vessels, pericytes are loosely attached to the endothelium and exhibit cellular modifications such as differential expression of typical markers and aberrant cytoplasmic projections that invade the tumor parenchyma [31–33].

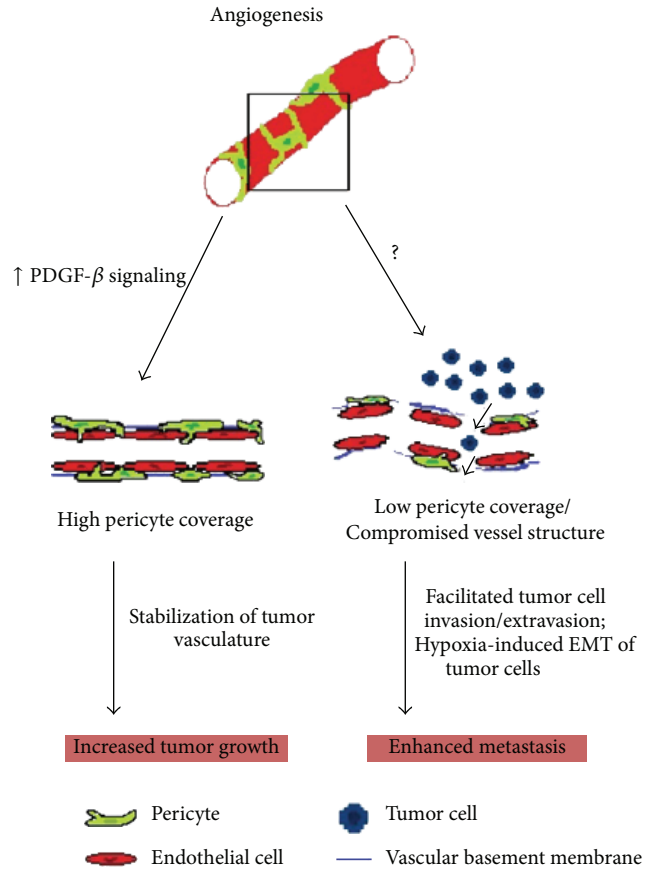

Figure 1: Abnormal pericyte coverage of tumor vessels affects tumor development. PDGF-β signaling controls pericyte recruitment during angiogenesis. Hyperactivation of this pathway within TME may increase pericyte coverage, thereby improving vasculature stability and perfusion, which favors tumor growth. In contrast, low pericyte coverage compromises vessel structure integrity, which becomes leaky, facilitating tumor cell invasion/extravasion. Under such circumstances, tumor cells may also undergo EMT induced by hypoxia, as a consequence of lower perfusion in the tumor vasculature. Both situations enhance metastatic spread of tumor cells.

Another abnormality often observed in tumor angiogenesis is the amount of pericyte coverage on tumor vessels, ranging from high to little or no coverage at all. Clinical studies have correlated the extension of pericyte coverage on tumor microvessels with cancer prognosis [34–37]. Increased pericyte coverage has been associated with tumors of melanoma and renal cell carcinoma with aggressive clinicopathological features, resistance to therapy, and unfavorable clinical outcome of patients [38]. In contrast, pericyte dysfunction or reduction has not been correlated with prognosis. Recent studies reported that pericyte ablation leads to increased vessel permeability and poor vessel integrity which, in spite of inhibiting tumor growth, favors blood vessel invasion by tumor cells and ensuing metastatic spread [39, 40]. These findings illustrate the many facets of pericyte effects on tumor angiogenesis (Figure 1).

It is still unclear why tumor vessels are not able to achieve proper pericyte coverage. One important mechanism of EC-pericyte communication involves the PDGF-β signaling, which is known to control pericyte migration during tumor angiogenesis [41]. In such mechanism, activated ECs produce PDGF-β, recruiting pericytes expressing PDGF-β receptors [22, 42]. In turn, pericytes stabilize the neovessels and contribute to ECs survival by locally releasing trophic factors, such as VEGF and Ang-1 [43, 44]. Blockage of pericyte recruitment by PDGF-β pathway inhibition leads to EC loss and subsequent regression of tumor vessels [30, 45]. Overexpression of PDGF-β, on the other hand, increases pericyte coverage, improves vessel stability, and accelerates tumor growth rates [46, 47]. Due to its relevance, therapies targeting pericyte recruitment have been considered. Other mechanisms governing pericyte migration have been covered in a recent review [48].

Pericytes have been shown to be capable of providing a scaffold of preexisting blood vessels for rapid revascularization of tumors after interruption of therapies that eliminates only ECs [49]. It seems that the remaining pericytes participate in a strategy developed by tumors to evade antiangiogenic therapies. Consequently, the combination of anti-VEGF and anti-PDGF therapies has been proposed and was shown to induce tumor vessel regression [50, 51]. More recently, treatment with anti-OLFML3 (olfactomedin-like 3) was reported to be significantly effective in reducing tumor vascularization, pericyte coverage on tumor vessels, and tumor growth [52].

Therefore, in addition to their role in tumor angiogenesis, the involvement of pericytes in vessel cooption, an important alternative pathway by which tumors obtain blood supply through the use of preexistent vessels, supports the development of novel antiangiogenic strategies targeting, not only ECs as usual, but also pericytes. The proposal that interaction of pericytes with tumor cells may determine the perivascular location of tumor propagating cells [53] provides further arguments to the relevance of pericytes in tumor development, although details of this phenomenon remain to be determined.

3. Metastasis

Dissemination of cancer cells to distant organs requires their survival through a challenging route beginning in the primary tumor site. Invasion into surrounding vessels or tissues, survival in a hostile environment (e.g., blood circulation), and ability to seed and recapitulate tumor growth in a new site are the main limiting steps in this process. All stages can be highly influenced by nonmalignant cells within the tumor microenvironment, including pericytes.

Although the initial studies of pericytes and tumor development were mostly focused on angiogenesis, showing that blockage of pericyte recruitment or function leads to reduced tumor growth due to compromised vessel structure and blood supply [26], later studies surprisingly revealed that loss of pericyte coverage facilitates tumor cell spreading.

One of the first evidences showing that pericytes may be negative regulators of metastasis was provide by Xian et al. (2006), using mice deficient in neural cell adhesion molecule (NCAM). In this landmark paper, they provide compelling evidence that destabilization of tumor vasculature due to detachment of pericytes and dysfunctional interaction with ECs leads to enhanced metastatic potential [54]. A previous study had already observed an enhanced metastatic frequency in knockout animals exhibiting compromised blood vessel structure [55]. Further clinical studies with colorectal and breast cancer patients corroborated this finding [35, 40]. Low pericyte coverage showed a significant correlation with distant metastasis and poorer survival. Similarly, in a xenograft model of prostate cancer, increased tumor cell invasion was associated with lower pericyte density on microvessels [56].

However, the underlying cellular and molecular mechanisms whereby pericytes may limit tumor metastasis have not been entirely elucidated. Pericytes may act as a physical barrier that makes the extravasation of tumor cells into the vessel lumen difficult and/or may actively promote metastasis by releasing factors that affect tumor invasion.

Alternatively, a recent proposal defends the idea that pericytes may be indirectly involved in tumor cell escape. They hypothesize that pericyte depletion originates leaky vessels which increases intratumoral/interstitial plasma volume and elevates local pressure. The higher fluid pressure favors compression of remaining tumor vessels, decreasing the blood flow and reinforcing hypoxia, which may trigger tumor metastasis through a hypoxia-induced epithelial-mesenchymal transition (EMT) mechanism [40]. In fact, recovery of tumor vascular integrity by improving ECs junctions and increasing pericyte coverage was effective in reducing leakage and enhancing perfusion. In melanoma models, normalization of tumor vessels was able to attenuate hypoxia and decrease EMT of tumor cells, resulting in inhibition of lung and lymph node metastasis [57].

Pericytes have also been suggested to contribute to the metastatic process by affecting the colonization and growth of tumor cells at distant sites [9]. The contact of tumor cells with microvessel walls in a pericyte-like position seems to be determinant in the successful extravasation and proliferation of melanoma and lung carcinoma cells in the brain [58]. Studies with two murine models of lung metastasis showed that the administration of sunitinib, a clinically approved antiangiogenic drug, led to pericyte depletion in the seeding location [59]. Interestingly, tumor cells were preferentially retained in the lung vasculature area displaying lower pericyte coverage. The suggested hypothesis is that pericytes may limit seeding at the target site, controlling and regulating the metastatic niche.

Moreover, endothelial-derived factors have been reported to influence breast cancer cell growth at sprouting vessels in metastatic sites [60]. Taken together, these findings support the emerging idea that microvascular cells, including pericytes, may affect metastasis establishment and tumor cell growth at secondary sites.

4. Stemness

The multipotent differentiation capacity of pericytes has long been proposed. In 1978, Meyrick and Reid had already demonstrated that pericytes were plastic cells, capable of developing into vascular smooth muscle cells (vSMCs) under hypoxic stress [61]. Differentiation into other nonvascular cells, primarily bone cells, was later described [62]. Subsequently, several studies described that pericytes obtained from a variety of tissues could differentiate into adipocytes, chondrocytes, and skeletal myofibers [63, 64]. Furthermore, pericytes derived from brain capillaries were also reported to be capable of converting into neural cell lineages [65]. These and several other evidences support the hypothesis of the perivascular zone as the *in vivo* niche of mesenchymal stem cells (MSCs) and pericytes being the MSC precursors [66].

Indeed, besides multipotency, pericytes and MSCs share other similarities, including expression of common cellular markers. While pericytes express surface antigens typical of MSCs, such as CD44, CD73, CD90, and CD105, MSCs also express pericytes markers, including NG2, Sca-1, α-SMA, and PDGFβ-R [15], suggesting a shared ontogeny. Both cell types also present similar homing properties. Pericytes and MSCs can proliferate and migrate in response to chemotaxis and damage signals, such as those occurring during wound healing and tumor development.

Some recent findings, however, indicate that not all pericytes display stem cell potential, such as the highly differentiated pericytes found in some large and small vessels [67, 68]. Other studies also suggest that pericytes may be a subpopulation of specialized MSC residing in perivascular locations, given that the pericytic behavior is not an intrinsic ability of all MSCs [68].

There are also growing evidences of pericytes with stemness potential in several central nervous system pathologies. Compared with other tissues, the pericyte number and coverage in brain capillaries are relatively higher, and they are crucial to the integrity and function of the blood-brain barrier. Pericytes have been considered as an alternative stem/progenitor cell reservoir within the brain, since they were shown to migrate, proliferate, and even differentiate in neural cells, in response to tissue injury, stress, and inflammation [65, 69, 70].

In brain cancer, the perivascular niche is critical to the maintenance of a stem cell-like state in tumor cells. Interaction of perivascular cells with cancer stem cells (CSC) was shown to regulate self-renewal and differentiation of the latter cells, which are strongly related to tumor aggressiveness [71]. Notably, in Glioblastoma, the most frequent and aggressive type of primary brain tumor, a contact-dependent interaction with tumor cells, switches on the tumor-promoter character of pericytes, inducing their participation in tumor initiation and progression [53].

Another surprising connection between pericytes and brain CSC was revealed by Cheng et al. (2013) [72]. The authors demonstrated that most pericytes residing in the perivascular niche of Glioblastoma are generated by CSC. Through a close interaction with vascular components, Glioblastoma stem cells are also able to diferentiate into functional endothelial cells [73]. These findings reveal an interesting reciprocal interaction between pericytes and CSC, favoring tumor development (Figure 2).

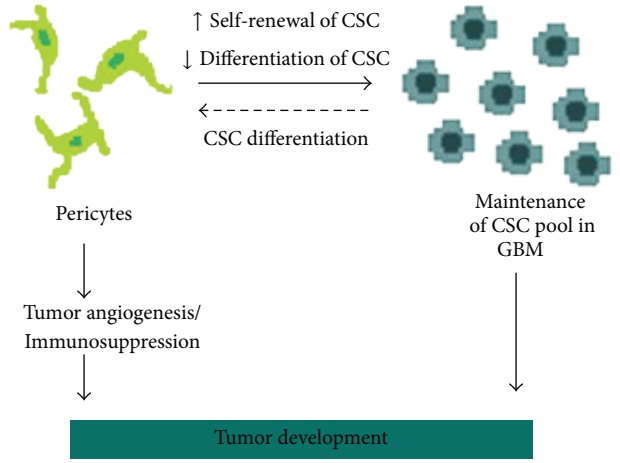

Figure 2: Interplay between pericytes and cancer stem cells. In brain cancer, the perivascular niche is critical to the maintenance of CSC pool. Perivascular cells promote self-renewal and impair differentiation of CSC. In turn, new pericytes may be generated by CSC, contributing to tumor angiogenesis and tumor scape from immune destruction. This reciprocal interaction between pericytes and CSC is highly beneficial to tumor development.

Based on the MSC properties of pericytes, Appaix et al. [74] also proposed that neoplastic pericytes in brain capillaries could be activated and recruited in response to inflammation signals, similar to what occurs during tissue regeneration. These neoplastic pericytes would then acquire a neural stem cell-like phenotype in the brain parenchyma and generate a pool of CSC, fueling tumor development. New pericytes generated from CSC could either contribute to tumor vascularization or restart the cycle. Due to their multipotency, pericytes could also generate other stromal cells constituting the TME. In fact, pericytes have been shown to differentiate into collagen-producing fibroblasts [75] and myofibroblasts [76], two major components of the heterogeneous population of cancer-associated fibroblasts. Although plausible, further experimental evidences are needed to support this model of neoplastic pericytes as tumor initiating cells.

In addition to inflammation signals, hypoxia is another important extrinsic factor within the TME that may recruit pericytes. Interestingly, brain-derived pericytes have been recently reported to generate neurovascular cells and activated microglial cells under hypoxic conditions [77, 78]. In gliomas, the most frequent group of primary central nervous system tumors, microglial cells are known to be recruited to the TME and activated to support tumor growth [79, 80]. Altogether, these evidences support an important role of pericytes as precursor cells for other stromal cells within the TME.

5. Immunomodulation

Tumor cells can evade the immune system through different mechanisms, some of which involving multiple cellular

components and immunosuppressive factors (e.g., TGF-β, prostaglandin E2, and interleukin-10) from the TME. Although the contribution of pericytes in this process is still elusive, recent data support pericytes as potential targets in cancer immunotherapy approaches.

Similar to MSCs, pericytes produce cytokines, chemokines, growth factors, and adhesion molecules that regulate immune cells under certain conditions. Several genes encoding immune factors in pericytes have been reported to be upregulated by activation of the PDGF-β signaling pathway [81], whose involvement in pericyte migration during angiogenesis was described above. In fact, pericytes have been considered an important component of the immunologic defense mechanism in the mammalian central nervous system [82, 83], where they were reported to express typical macrophage markers, such as ED-2, CD11b, CD68, and MHC class II, and exhibit immune cell properties, such as phagocytic and antigen-presentation activities [14, 84].

Brain pericytes, in particular, are highly sensitive to inflammatory stimuli and may differentially respond according to the cytokine involved. Studies with porcine brain capillary pericytes reported a rapid upregulation of iNOS and COX-2 mainly after stimulation with interleukin-1 beta (IL-1β). Upregulation of iNOS was accompanied by increments in the intracellular oxidative status of pericytes. The same study also reported induction of phagocytosis of opsonized particles and MHC II expression in pericytes by tumor necrosis factor-alpha (TNF-α) or interferon-gamma (IFN-γ) treatment, characteristic of an antigen-presentation activity [84].

Interaction between pericytes and immune cells also occurs during tissue repair, when pericytes may actively participate in leukocyte recruitment and diapedesis. Using an experimental model of brain inflammation, Pieper et al. [85] demonstrated that treatment with TNF-α, IL-1β, or LPS stimulates secretion of IL-8 and matrix metalloprotease-9 by brain pericytes, facilitating chemoattraction and transmigration of neutrophils.

However, in the cancer context, there are evidences that maturation of pericytes and restoration of the normal tumor vasculature improve transmigration of immune cells into tumors. A study with the RIP1-Tag5 mouse model of pancreatic islet carcinogenesis showed that deletion of the *Rgs5* gene, encoding a regulator of G-protein signaling with expression restricted to pericytes in the vascular tissue, induced changes in the vasculature and enhanced infiltration of $CD8^+$ T lymphocytes in tumors. As a consequence, the immune-mediated tumor rejection was exacerbated, resulting in improved survival of tumor-bearing mice [86].

In agreement with these observations, Bose et al. [87] reported upregulation of *Rgs5* in murine pericytes when these cells were cocultured with fragmented tumor cells or were directly injected into established tumors *in vivo*. Moreover, tumor-derived pericytes were able to induce CD4+ T cell anergy and this effect was rescued after *Rgs5* silencing. Interestingly, in addition to *Rgs5*, upregulation of *PDL-1* was also observed in pericytes cultivated in the presence of tumor fragments. Since PDL-1 expression in cancer cells is known to inhibit the activity of PD-1+/CD8+ T cells [88], the combined effects of RGS5 and PDL-1 expression in pericytes may improve protection of tumor cells from T cell-mediated death. Indeed, pericytes isolated from human malignant gliomas, characterized by coexpression of CD90, PDGFR-β, and CD248, were also suggested to have immunosuppressive properties within the TME, based on their capacity to inhibit proliferation of cytotoxic T lymphocytes [89].

These findings point out important direct and indirect effects of pericytes in the immune response against tumor cells, whose underlying mechanisms remain to be fully dissected.

6. Conclusions

An overall analysis of the functional properties of pericytes reveals that these are multifaceted cells with ability to significantly influence tumor development. As a component of the TME, pericytes may actively contribute to some classic cancer hallmarks, namely, induction of angiogenesis, sustained tumor growth, metastasis, and evasion of immune destruction.

Disruption of the delicate balance of pericyte coverage on tumor vessels seems critical since it may either induce tumor growth or facilitate metastatic spread. The interplay between pericytes and CSC is also compatible with the updated dynamic CSC model. Pericyte-mediated regulation of stemness properties in cancer cells could help maintain a residual CSC pool, whose cell progenies include both tumor and stromal cells. The immunosuppressive phenotype acquired by pericytes once in the TME is also of great relevance since they may act in synergy with tumor cells to inhibit local immune response. This scenario is highly favorable to current cancer immunotherapy strategies, such as the use of monoclonal antibodies targeting the PD-1/PDL-1 signaling.

Altogether, this analysis argues in favor of pericytes as cellular targets for new cancer therapies aiming at the TME. Modern cancer treatments largely rely on such strategy, with antiangiogenic and immunosuppressive drugs as the main examples. Development of new drugs addressing pericytes would have the advantage of targeting multiple cancer hallmarks at once, increasing the chances of treatment efficacy. However, given the prometastatic effects of pericyte depletion on tumor vessels, development of such therapeutic strategy is not straightforward and should be more beneficial to early-stage diseases or to tumors with low metastatic potential. As phenotypic and functional characterization of pericytes progresses, particular subtypes of pericytes may also emerge as clearer targets for therapeutic purposes, such as the case of type-2 pericytes which are specifically recruited during tumor angiogenesis. Ultimately, pericyte-targeted therapies should be tested in combination with other treatment modalities to address possible synergistic effects aiming at meaningful tumor regression without favoring metastatic spread.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

The Role of Adipose-Derived Stem Cells in Breast Cancer Progression and Metastasis

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Conventional breast cancer extirpation involves resection of parts of or the whole gland, resulting in asymmetry and disfiguration. Given the unsatisfactory aesthetic outcomes, patients often desire postmastectomy reconstructive procedures. Autologous fat grafting has been proposed for reconstructive purposes for decades to restore form and anatomy after mastectomy. Fat has the inherent advantage of being autologous tissue and the most natural-appearing filler, but given its inconsistent engraftment and retention rates, it lacks reliability. Implementation of autologous fat grafts with cellular adjuncts, such as multipotent adipose-derived stem cells (ADSCs), has shown promising results. However, it is pertinent and critical to question whether these cells could promote any residual tumor cells to proliferate, differentiate, or metastasize or even induce *de novo* carcinogenesis. Thus far, preclinical and clinical study findings are discordant. A trend towards potential promotion of both breast cancer growth and invasion by ADSCs found in basic science studies was indeed not confirmed in clinical trials. Whether experimental findings eventually correlate with or will be predictive of clinical outcomes remains unclear. Herein, we aimed to concisely review current experimental findings on the interaction of mesenchymal stem cells and breast cancer, mainly focusing on ADSCs as a promising tool for regenerative medicine, and discuss the implications in clinical translation.

1. Introduction

Breast cancer is the most-frequently diagnosed cancer and a leading cause of cancer-related death in women worldwide [1–3]. Great effort has been put into pursuing the understanding of breast cancer development, progression, and invasion, as well as implementation of appropriate therapies. Depending on breast cancer stage, therapy may include chemotherapy, irradiation, and, most frequently, surgical treatment ranging from local excision and lumpectomies to modified and radical mastectomies. Oncological surgery is disfiguring and the original anatomical contours of the breast often require reconstitution. Besides the use of synthetic prosthetics or flap surgery, a more recent alternative for restoring the breast shape and camouflaging scars is transplantation of autologous lipoaspirates, referred to as "lipofilling" or "fat grafting." Ideally, autologous fat transplantation has the advantage of providing a more natural appearance after reconstruction, in addition to being readily available tissue coupled with low donor-site morbidity from liposuction as compared to flap surgery [4]. However, long-term outcomes are unpredictable in terms of engraftment of transplanted fat aliquots, as there is a variable loss of volume, which often dictates unsatisfactory final outcomes and the necessity for repetitive lipofilling sessions [5–7]. The reason has mainly been attributed to poor vascularization of fat grafts with consequent fat necrosis and/or apoptosis [5]. To overcome this drawback, supplementation with adipose-derived stem cells (ADSCs) isolated from white adipose tissue (WAT) has been proposed, which is believed to improve fat engraftment

[5, 7–9] and have additional positive effects on scars and damaged skin after irradiation therapy [10, 11]. These cells are incorporated in the autologous fat graft but can be isolated to further enhance the regenerative potential of smaller volume injections.

ADSCs share similarities with mesenchymal stromal cells (MSCs) isolated from bone marrow (BM-MSCs) [12]. Through cytokine and growth factor release, ADSCs have shown several beneficial effects in inflammatory and autoimmune diseases and ischemic conditions [13–15]. Moreover, inherent advantages over MSCs isolated from other tissues, such as higher yields and lower harvest site morbidity [16], as well as their natural relation to WAT itself, make ADSCs an ideal tool for soft tissue reconstruction. Early reports show beneficial effects of ADSCs on autologous fat grafting with improved retention rates when coinjected [4, 9, 17–19].

MSCs are able to home to sites of tissue injury and inflammation [20], as well as the cancer microenvironment (CME) [21, 22]. In this regard, some authors proposed the use of MSCs either as a vector for anticancer therapy or as an adjunct treatment for increasing cancer cell susceptibility to chemotherapies [23, 24].

However, both BM-MSCs and ADSCs are also suspected to promote tumor development and progression, as well as recurrence in different cancer types [25–27]. MSCs in general have controversially been reported to support [26, 28–31] or to suppress [32–34] cancer cells. Thus, considering the fact that the risk of breast cancer recurrence is up to 13% after adjuvant therapy [35], investigating the effects of ADSCs on breast cancer prior to performing ADSC-enhanced fat grafting for reconstructive purposes after oncological surgery on a routine basis is of the utmost importance.

Several mechanisms have been proposed through which ADSCs, and more in general MSCs, interact with cancer cells and influence their microenvironment. These include paracrine signaling and cell-to-cell signaling, as well as differentiation into cancer-associated myofibroblasts (CAFs) or incorporation into newly formed vessels, leading to morphological and functional alterations of both cancer cells and MSCs in a bidirectional manner and the cancer niche itself [36–38]. Furthermore, several reports on the interaction between ADSCs and breast cancer cells (BCCs) have been published [30, 38–43].

The use of ADSCs for reconstructive purposes after breast cancer surgery has gained attention in recent years. The effects of ADSCs, which might improve fat retention after soft tissue reconstruction, potentially could be beneficial for the survival and promotion of residual cancer cells, a sort of "double-edged sword." In this review, we will focus on the influence of ADSCs on BCCs and concisely summarize different putative mechanisms potentially involved in promotion and spread of breast cancer and discuss the difference to actual clinical findings.

2. Adipose-Derived Stem Cells

Just over a decade ago, Zuk et al. reported a multipotent, undifferentiated, self-renewing progenitor cell population isolated from WAT that is morphologically and phenotypically similar to BM-MSCs [12]. ADSCs were found to be able to differentiate into a variety of mesenchymal lineages including adipogenic, osteogenic, chondrogenic, and hepatocytic differentiation [12]. Through paracrine secretion of a broad selection of cytokines, chemokines, and growth factors, ADSCs have been shown to have antiapoptotic, proangiogenic, anti-inflammatory, immunomodulatory, and antiscarring effects. This potential makes them promising candidates for cellular therapy in regenerative medicine [9, 15, 44, 45]. Unlike bone marrow, fat is abundantly available and easily accessible through liposuction and can yield significantly higher amounts of cells, which makes adipose-derived cells appealing for regenerative medicine [14].

2.1. Sources and Subpopulations of ADSCs. The most common source for ADSCs is abdominal fat [23, 46, 47], as well as breast tissue, either after reduction mammoplasty [42, 48, 49] or after breast cancer surgery [50, 51]. The surgical technique and the back-table processing after harvesting are not discussed in detail in the reviewed papers. Thus, any comparisons between studies that will facilitate standardization of such parameters remain a challenge. It has been shown that the anatomical location of harvest can influence proliferation and function [52], differentiation ability [53], and apoptotic susceptibility [54] of ADSCs. For example, ADSCs derived from superficial abdominal fat depots [54] are more resistant to apoptosis, which might be relevant to ADSC survival in the highly active tumor microenvironment. Indeed, ADSCs from different anatomical regions (e.g., inguinal, omental, and pericardial) have been found to express different surface marker patterns [55] and the cell yields of ADSCs also vary by anatomical region of isolation [56].

Breast ADSCs seem to express similar surface marker phenotypes as abdominal ADSCs (positive for CD29, CD73, CD90, and CD105 and negative for CD14, CD31, CD34, and CD45) according to a recent report by Hanson et al. [57], although CD34 expression was found to differ between breast ADSCs isolated from cancer-affected mammary fat and normal breast fat tissue [58]. Regardless of the passage, ADSCs derived from normal breasts were $CD34^+$, in contrast to CD34-negativity in cancer afflicted breast tissue-derived ADSCs. This is in contrast to a recent report by Yang et al., which found only minimal expression of CD34 in normal breast-derived ADSCs [48]. Nevertheless, ADSCs from abdominal and normal breast fat share similar genetic profiles [59]. Moreover, reports of ADSCs isolated from primary breast cancer tissue have been published [50, 60–62].

After homogenization of whole fat or lipoaspirates, a pooled cell pellet, the stromal vascular fraction (SVF), remains. The SVF contains a heterogeneous population of cells that includes at least four subpopulations with distinct surface marker phenotypes, in addition to erythrocytes and lymphocytes, namely, endothelial progenitor cells (EPCs; $CD45^-CD31^+CD34^+$), mature endothelial cells (ECs; $CD45^-CD31^+CD34^-$), pericytes ($CD45^-CD31^-CD34^-CD146^+$), and supra-adventitial ADSCs ($CD45^-CD31^-CD34^+$) [63, 64]. Adipose-derived pericytes

Table 1: Most common human BCC lines used for investigation of ADSC/breast cancer interaction [68, 69, 191].

BCC line	Classification	ER	PR	Her2	In culture	Notes	References
MCF-7	Luminal A	+	±	−	Mass	Endocrine responsive Isolated from MPE	[36, 42, 46, 47, 50, 58, 61, 70–73, 98, 109]
MDA-MB-231	Basal B, claudin-low	−	−	−	Stellate	Isolated from MPE	[22, 30, 36, 38, 42, 49, 71–74, 89, 109]
T47D	Luminal A	+	±	−	Mass	Endocrine responsive Isolated from MPE	[70, 73, 93]
BT-474	Luminal B	+	+	+	Mass	Endocrine and Trastuzumab responsive Isolated from primary tumor	[70, 71]
HCC1937	Basal A	−	−	−	n/a	Isolated from primary tumor	[41, 43]
MDA-MB-436	Basal B	−	−	−	Stellate	Isolated from MPE	[41, 43]
ZR 75.1	Luminal B	+	±	+	Grape-like	Endocrine and Trastuzumab responsive. Isolated from ascites	[39, 41]
SKBR3	Luminal, Her2	−	−	+	Grape-like	Trastuzumab responsive Isolated from MPE	[23]
T4-2 (HMT-3522)	Basal B	−	−	−	Mass	Isolated from primary tumor	[74]

BC: breast cancer; BCC: breast cancer cell; ER: estrogen receptor; Her2: human epidermal growth factor receptor 2; MPE: metastatic pleural effusion; n.s.: not specified; PR: progesterone receptor.

are rare (~1% of SVF) and are thought to be a progeny for the less primitive ADSCs, which express mesenchymal surface markers such as CD73, CD90, and CD105, but also CD34 [65]. However, the International Society for Cell Therapy (ISCT) definition for plastic adherent MSCs clearly includes the absence of CD34 [66]. Nevertheless, both pericytes and ADSCs have excellent adipogenic differentiation potential, which makes them both ideal cells for reconstructive purposes [63, 64]. $CD34^+$ cell prevalence in fat grafts correlates with extent of graft retention and shows individual variability among patients [9]. In a joint statement paper from the International Federation for Adipose Therapeutics and Science (IFATS) and the ISCT, ADSCs were defined as a $CD34^+$ subpopulation of the SVF [67].

Among the different studies investigating ADSCs and BCCs, there is a consensus that ADSCs express mesenchymal surface markers such as CD29, CD44, CD73, CD90, and CD105 and lack hematopoietic and endothelial markers (e.g., CD31, CD45). However, reports of CD34 expression are conflicting, with different authors naming "ADSC" cell populations either with or without CD34 expression. One should keep this in mind when comparing experimental results, since different expressions of CD34 could mean different cell subpopulations, in addition to the potential effects of culture on surface marker phenotype switch or loss [51]. Different cell subpopulations of the SVF are likely to share similarities and overlap in some surface marker expression but might have slightly different differentiation potential and/or functional characteristics [44]. The translational relevance of CD34 expression currently remains unclear.

In this review, ADSCs will be generally termed as those adipose-derived cells which are plastic adherent and can be expanded in culture after isolation of the SVF. This comprises cells that present a heterogeneous expression of CD34 but express unquestionable mesenchymal markers (e.g., CD29, CD44, CD73, CD90, and CD105) and lack hematopoietic and other endothelial markers (e.g., CD31, CD45).

3. Breast Cancer Cell Lines

Table 1 gives an overview of the BCC lines most commonly used for experimental studies on ADSCs and breast cancer interaction [68, 69]. Mainly, most of the experiments make use of human cell lines in humanized murine (xenotransplant) models *in vivo*. MCF-7 is the most common cell line used, especially to assess driving mechanisms of breast cancer progression from a relatively low malignancy to an invasive and metastatic phenotype [36, 42, 46, 47, 50, 70, 71]. The MDA-MB-231 line, on the other hand, is mostly used to investigate metastatic spread and basic biology of aggressive breast cancers [22, 30, 42, 72–74]. The different cell lines have distinct characteristics in culture and *in vivo* and may be used for specific research aims, so MCF-7 and BT-474 cells are ideal for investigation of hormone-receptor roles and their associated therapeutic approaches; ZR75.1 and SKBR3 cells, both HER-2 positive, might be used for testing therapies similar to trastuzumab; MDA-MB-231 and MDA-MB-436 are used for research on "triple-negative," basal-like breast cancers [69].

Many of these cell lines were isolated many decades ago and were immortalized, with changes to both gene expression and phenotype over time as a potential consequence. A number of commonly used cell lines such as MCF-7 were isolated from metastatic pleural effusions (MPEs) and might not depict the most common tumor biology but an advanced one, due to originating from a metastatic cancer.

4. Interactions between Adipose-Derived Stem Cells and Breast Cancer Cells

4.1. ADSC Homing and Migration. There is evidence that MSCs home to injured tissue, sites of inflammation, and tumor niches [20, 21, 75]. This has been shown *in vivo* when administered intravenously and also for endogenous MSCs [76]. Tumor irradiation also promotes MSC recruitment into the irradiated area, probably due to induced tissue inflammation [77] or the necessity for tissue repair. Due to their inherent ability to home to cancer tissue as well as hinting to sensitizing cancer cells for chemotherapy, MSCs have also been proposed as vehicles for targeted anticancer drugs or gene therapy [49, 78, 79].

There are a multitude of surface signaling molecules, cytokines, and chemokines that are able to induce and control MSC recruitment and migration from their physiological niches and their homing into the injured tissues and cancer. Granulocyte colony stimulating factor and granulocyte-macrophage colony stimulating factor are two of the most well-known factors widely used for stem cell mobilization in the clinical setting [80, 81]. Stromal-derived factor 1 (CXCL-12) and its receptor CXCR-4 are also key players in cellular homing [82, 83] and have been shown to be involved in MSC migration, in addition to having an important role also in (tumor) angiogenesis [84]. Other molecules, for example, vascular cell adhesion molecule 1, MCP-1, and MMPs, are also involved in the complex and multifactorial MSC homing process [85–87].

ADSCs as a component of WAT are physiologically located in the breast and potentially near any occurring breast cancer. Moreover, additional ADSCs could be inoculated through reconstructive cell-assisted lipografting close to the cancer bed. This is different than BM-MSCs, which must be recruited through mobilization from the bone marrow into circulation and home to cancer. An interesting study by Kidd et al. suggests that mobilization from both fat and bone marrow may be induced by breast cancer, with the two cell types playing distinct roles in the CME [76]. *In vitro*, ADSCs have been found to migrate towards conditioned medium (CM) of both MDA-MB 231 and 4T1 breast cancer cells [88].

Karnoub et al. observed homing of intravenous-applied MSCs to the tumor niche, with no evidence of accumulation in filtering organs [26]. This agrees with another work showing viable GFP^+ ADSCs in breast cancer tumors after two weeks in a perivascular location [89] after homing. Other reports indicate substantial engraftment of human MSCs in the liver in addition to being present in tumor tissue for weeks [90]. Regardless of local or intravenous delivery, they promoted both tumor growth and invasiveness [30]. Direct coinjection of ADSCs and BCCs increased growth to a higher extent, suggesting a partial entrapment of injected cells in filtering organs (e.g., lungs, spleen, and liver). ADSCs within the tumor survived for at least 20 days and were found to differentiate into ECs and incorporate into new cancer-associated vasculature. In Karnoub's study, metastases were increased under the influence of MSCs for several BCC lines, including high malignant MDA-MB-231 and low malignant MCF-7 cells. This effect was abolished when MSCs were injected in the mammary pad contralateral to developing breast cancer, unlike results of another report, where, interestingly enough, cells injected subcutaneously were able to home to the tumor site on the contralateral mammary pad through blood circulation [89], underscoring the ability of MSCs to home to sites of tissue damage following different paths.

4.2. Cancer Promotion and Suppression. Studies investigating the impact of ADSCs, and more in general MSCs, on cancer growth dynamics and patterns, as well as progression to metastatic disease, revealed somewhat contradictory results, showing both promoting and suppressing effects. Tables 2 and 3 summarize the most important experimental *in vitro* and *in vivo* studies, respectively.

In their 2007 study, Karnoub et al. reported that BM-MSCs promote the disposition of BCCs to migrate when cocultured with low malignancy cell lines such as MCF-7 [26]. A number of preclinical studies followed, suggesting that BM-MSCs can exert a promoting influence on the growth and spread of breast cancer [29, 31, 91, 92]. In 2009, the first reports showing similar cancer-promoting effects with the use of ADSCs were published, depicting that the issue might extend to MSCs coming from different sources as well [38, 73]. In a similar fashion, Kucerova et al. found that BM-MSCs and ADSCs promoted proliferative effects in a variety of BCC lines [93], but not on SKBR3 [23]. This was in line with reports from a Chinese group, which found decreased tumor proliferation with high numbers of MSCs [34, 94]. Sun et al. also showed that human BM-MSCs and ADSCs homed to tumors and were able to inhibit growth of high malignancy MDA-MB-231 cells and decrease metastatic spread of a normally migratory cell line *in vivo* [49]. These findings were confirmed in later studies by the same group with both umbilical cord-derived MSCs and ADSCs injected simultaneously with or three weeks after inoculation of BCCs [95]. This might be a very important finding, as the timing might more appropriately reflect the clinical scenario of stem cell-enhanced autologous fat grafting.

Rowan and colleagues discovered that ADSCs did not increase proliferation in triple-negative BCCs but did slightly in hormone-receptor positive cells such as MCF-7 and BT-474. On the other hand, not only the *in vitro* migration potential of triple-negative MDA-MB-231 was enhanced by ADSCs, but also their CM was enough to achieve similar results, suggesting a paracrine mechanism [71]. These results are similar to those observed with the bone marrow-derived counterpart in the earlier report by Karnoub et al. [26].

Of WAT-derived cells, $CD34^+$ cells seem to be at least partly responsible for tumor-promoting ability, as they increased tumor sizes significantly when coinjected with BCCs. In addition, $CD34^+$ cells seem to be more efficient in a metastatic shift of triple-negative MDA-MB-436 and HCC1937 cells in a murine xenograft model [43]. In a study published later by the same group, two distinct $CD34^+$ populations were found to act in concert when promoting breast cancer growth [41]. EPCs promoted neovascularization to a higher extent and were more prone to migration into lymph

Table 2: Relevant *in vitro* studies investigating the effects of ADSCs on breast cancer.

Reference	Year	ADSC origin	ADSC surface marker	BCC line	Effects on BCC
Trivanović et al. [58]	2014	Human breast (normal versus cancer-affected) and abdominal	$CD44^+CD73^+CD90^+CD105^+$ $CD11a^-CD33^-CD45^-CD235a^-$ $HLA\text{-}DR^-CD34^\pm$	MCF-7	Proliferation↑ (direct coculture) Proliferation↓ (indirect coculture) Different ADSCs had similar effects
Kucerova et al. [23]	2013	Human lipoaspirates	$CD29^+CD44^+CD90^+CD105^+$ $CD14^-CD34^-CD45^-$	SKBR3	Proliferation↓, migration↑ EMT markers↑ BCC chemosensitivity↑
Lin et al. [46]	2013	Human lipoaspirates	$CD29^+CD44^+CD105^+$ $CD31^-CD34^-HLA\text{-}DR^-$	MCF-7	Proliferation and migration↑ Cell-to-cell contact needed Wnt pathway↑
Strong et al. [72]	2013	Human abdominal versus nonabdominal	n.s.	MCF-7, MDA-MB-231	Proliferation↑ Leptin/estrogen-dependent Increased effect of abdominal ADSCs from obese (versus lean and nonabdominal ADSCs)
Zhang et al. [50]	2013	Human breast (cancer-affected)	$CD13^+CD29^+CD44^+CD71^+$ $CD105^+HLA\text{-}I^+$ $CD4^-CD10^-CD14^-CD34^-$ $CD38^-HLA\text{-}DR^-$	MCF-7	Proliferation↑ Migration↑
Zhao et al. [47]	2013	Human lipoaspirates (abdominal)	$CD29^+CD44^+CD105^+$ $CD34^-CD45^-$	MCF-7	Migration↑ Angiogenesis↓ MMPs↑
Devarajan et al. [70]	2012	Human whole fat	n.s.	4T1 (murine) BT-474, MCF-7, T47D	Proliferation↑, EMT markers↑ PDGF-dependent (paracrine)
Jotzu et al. [109]	2011	Human whole fat	$CD29^+CD44^+CD90^+CD105^+$ $CD14^-CD34^-CD45^-$	MCF-7, MDA-MB-231	Migration and invasion↑ ADSCs differentiate to CAFs
Kucerova et al. [93]	2011	Human lipoaspirates	$CD44^+CD73^+CD90^+CD105^+$ $CD14^-CD34^-CD45^-$	MCF-7, T47D, MDA-MB-361	BCC proliferation↑ (dose-dependent) Paracrine mechanism
Razmkhah et al. [61]	2011	Human breast (cancer-affected)	$CD44^+CD105^+CD166^+$ $CD14^-CD34^-CD45^-$	MCF-7	Anti-inflammatory cytokines↑ T regs↑
Yan et al. [98]	2012	Human breast (normal versus cancer-affected)	$CD29^+CD73^+CD90^+CD105^+$ $CD166^+$ $CD31^-CD144^-CD14^-CD45^-$ $HLA\text{-}DR^-$	MCF-7	Proliferation↑ (BC ADSCs > normal breast ADSCs) EGF/EGFR/Akt-dependent
Pinilla et al. [38]	2009	Human abdominal	n.s.	MDA-MB-231	Proliferation↑, RANTES↑ Migration↑, MMPs↑
Welte et al. [73]	2012	Human lipoaspirates (abdominal)	$CD44^+CD90^+CD105^+$ $CD11b^-CD14^-CD34^-CD45^-$ $HLA\text{-}DR^-$	MCF-7, MDA-MB-231, T47D	ADSC migration towards BCCs Migration and invasiveness↑ IL-8↑

ADSC: adipose-derived stem cell; BC: breast cancer; BCC: breast cancer cell; CAF: cancer-associated (myo) fibroblast; EMT: epithelial-to-mesenchymal transition; ER: estrogen receptor; Her2: human epidermal growth factor receptor 2; MMPs: matrix metalloproteinases; MPE: metastatic pleural effusion; n.s.: not specified; PR: progesterone receptor; T reg: regulatory T lymphocyte.

nodes and metastasis formation, whereas ADSCs locally promoted tumors more than EPCs. Strikingly enough, $CD34^-$ cells promoted growth to a lesser extent, and metastases were similar to controls without WAT cells. Ironically, the $CD34^+$ subpopulation is the one which shows high benefits for retention of fat grafts and therefore would be an appealing tool for reconstructive efforts [9].

Noteworthily, two published papers by Ke et al. and Zimmerlin et al. included *in vivo* models in which they seeded cancer cells in numbers as low as ten and 100 cells, respectively [5, 96]. The first group showed that ten murine 4T1 breast cancer cells (low malignancy) were able to grow into a tumor and metastasize upon coinjection with murine BM-MSCs, whereas the same BCCs alone failed to do so [96]. The authors suggested increased angiogenesis, as depicted by enhanced vascularity next to GFP^+ BM-MSCs as one of the mechanisms. Interestingly enough, and in contrast to other studies, MSCs were not present in the tumor at later

Table 3: Relevant *in vivo* studies investigating the effects of ADSCs on breast cancer.

Reference	Year	Model	ADSC origin	ADSC surface markers	BCC line	Ratio BCC/ADSC	Effects on BCC/BC
Eterno et al. [60]	2014	Mouse	Human lipoaspirates and breast whole fat (normal versus cancer-affected)	$CD44^+CD90^+CD117^+$ $CD133^+$ $CD34^{low}CD45^-$	MCF-7, MDA-MB-231, primary BCCs	2 : 1	No changes in MCF-7 MDA-MB-231 growth and migration↑ EMT↑ Paracrine, IL-8↑, IL-6↓
Rowan et al. [71]	2014	Mouse	Human lipoaspirates (abdominal)	$CD29^+CD34^+CD73^+$ $CD90^+CD105^+$ $CD44^{low}CD45^{low}$	BT-474, MCF-7, MDA-MB-231	1 : 1	Tumor growth↔ Migration and metastasis↑ EMT induction
Orecchioni et al. [41]	2013	Mouse	Human lipoaspirates	$CD31^+CD34^+CCRL2+$ $CD13^-CD45^-$ (EPC) and $CD13^+CD34^+$ $CD140b^+$ $CD31^-CD45^-$ (ADSC)	HCC1937, MDA-MB-436, ZR75-1	5 : 1	Tumor growth↑ Metastatic spread↑ EMT↑ Effect of ADSCs > EPCs
Chandler et al. [36]	2012	Mouse	Human lipoaspirates	$CD13^+CD29^+CD44^+$ $CD73^+CD90^+CD105^+$ $CD166^+$ $CD14^-CD31^-CD45^-$	MCF-7, MDA-MB-231	1 : 1	Tumor growth↑ Angiogenesis↑ Bidirectional signaling ADSCs differentiate to CAFs
Zhang et al. [22]	2012	Mouse	Murine (endogenous)	$CD34^+$ $CD31^-CD45^-$	E0771, MDA-MB-231	n.s.	Circulating ADSCs↑ in cancer ADSCs incorporate into tumor vasculature (as pericytes)
Zhao et al. [74]	2012	Mouse	Human breast (normal)	$CD29^+CD73^+CD90^+$ $CD105^+$ $CD14^-CD31^-CD45^-$	HMT-3522 S3 (preinvasive), HMT-3522 T4-2 (invasive), MDA-MB-231	1 : 1, 3 : 2	Tumor growth↑ Tumor invasiveness↑ Angiogenesis↔ No effect on preinvasive BCCs
Dirat et al. [39]	2011	Mouse	Murine 3T3 adipocytes	—	4T1, 67NR, (murine) ZR 75.1, SUM159PT	n.s.	Metastatic spread↑ IL-6-dependent
Martin-Padura et al. [43]	2012	Mouse	Murine whole fat	$CD34^+$ $CD45^-$	HCC1937, MDA-MB-436	5 : 1	Tumor growth↑ Metastatic spread↑ Angiogenesis↑
Zimmerlin et al. [5]	2011	Mouse	Human abdominal whole fat	$CD34^+CD44^+CD73^+$ $CD90^+CD105^+CD146^+$ $CD45^-CD31^-$	Human MPE	n.s.	Tumor growth↑ (active cells, but not resting cells)
Muehlberg et al. [30]	2009	Mouse	Murine whole fat	$CD44^+CD90^+CD105^+$ $CD11b^-CD14^-CD34^-$ $CD45^-HLA\text{-}DR^-$	4T1 (murine), MDA-MB-231	1 : 10	Tumor growth↑ Metastatic spread↑ Paracrine through SDF-1 ADSCs home to tumor and differentiate to ECs
Sun et al. [49]	2009	Mouse	Human breast whole fat	n.s.	MDA-MB-231	2 : 1	Tumor growth↓ Metastatic spread↓ No early carcinogenesis improvement
Walter et al. [42]	2009	Mouse	Human breast whole fat and abdominal lipoaspirates	n.s.	MCF-7, MDA-MB-231	1 : 1	Tumor migration and invasiveness↑ IL-6-dependent
Zhang et al. [89]	2009	Mouse	Murine whole fat (obese mice)	$CD34^+$ $CD31^-CD45^-$ (ADSC) and $CD31^+CD34^+$ $CD45^-$ (EPC)	4T1, EF43.fgf4 (murine), MDA-MB-231	n.s.	Tumor growth↑ ADSCs home to tumor (perivascular space)

ADSC: adipose-derived stem cell; BC: breast cancer; BCC: breast cancer cell; EC: endothelial cell; EMT: epithelial-to-mesenchymal transition; EPC: endothelial progenitor cell; ER: estrogen receptor; Her2: human epidermal growth factor receptor 2; MMPs: matrix metalloproteinases; MPE: metastatic pleural effusion; n.s.: not specified; PR: progesterone receptor; T reg: regulatory T lymphocyte.

time points beyond 11 days [96]. Zimmerlin et al. isolated cells in different dormancy states: persistent, dormant cells after surgical therapy and active cells representing the active disease as a primary or recurrent tumor. The authors isolated mainly three cancer cell types, namely, small resting and large active cancer cells, both $CD90^+$ and a third $CD90^-$ population. Small resting cells were rare and represented only a small portion of the isolated cells. However, these cells may potentially lead to recurrence [5]. Combined with ADSCs, 100 small resting cells were not affected. The same aliquot of large cells was not capable of developing a cancer nodule but developed to a significant size when coinjected with ADSCs. These findings could be explained by the autonomy of slow-growing dormant cells, whereas active cells require a high amount of growth factors and good vascularity. This is in line with other findings in which breast ADSCs were able to promote the progression and invasion of the invasive cancer cell line T4-2, but not its preinvasive variant HMT-3522 S3 [74]. These results suggest that fat grafts supplemented with ADSCs for reconstruction could be used in patients after complete and terminated cancer-therapy and documented healing, since they may affect active but not resting cancer cells [5].

4.3. MSCs and the Cancer Microenvironment. Besides being a highly proliferative and dynamic mammary gland tissue, breast tissue contains a stroma with a heterogeneous cell population including adipocytes, myofibroblasts, MSCs, and ECs, as well as macrophages and other immune system cells [97]. Similarly, this stroma is actively involved in creating the CME, which is composed of highly proliferative malignant cancer cells and several nonmalignant elements including cancer-associated vessels, the extracellular matrix (ECM), CAFs [36, 76], stromal cells such as MSCs [98], and immune cells like macrophages and lymphocytes [99]. Emulating a chronic wound and secreting chemoattractant factors, tumors "trick" and attract MSCs from the bone marrow and possibly other locations such as local and peripheral fat [100].

The interaction between the stroma resident cells such as ADSCs and cancer-associated fibroblasts and primary cancer cells is sophisticated and happens in a bidirectional fashion, with the different cells influencing each other on different levels. MSCs that have homed to a tumor can have different fates: they may survive and exist as MSCs or differentiate into another cell type, such as ECs, pericytes, or CAFs [101–103]. MSCs and CAFs share similarities in regard to phenotype and surface markers, but CAFs additionally express fibroblast-specific protein and fibroblast activation protein, as well as α-SMA, and have been shown to produce higher levels of IL-4, IL-10, TGF-β1, and VEGF [104]. The basal-like $CD44^+CD90^+$ small cells at the stroma/tumor interface cross talk with surrounding CAFs, which provides an ideal niche for the growing tumor mass. Those cells later migrate to the inside of the tumor bulk and become highly proliferative $CD44^+90^-$ cells. Noteworthily, the $CD44^+90^+$ cells have been regarded as tumor cell progenitors and might serve as cancer stem cells (CSCs) [40].

BM-MSCs and ADSCs have been shown to differentiate into CAFs *in vivo* and *in vitro* [103, 105–109]. Kidd et al. found that CAFs originate mainly from endogenous bone marrow precursor cells, whereas progenitor cells from local adipose tissue are the origin of pericytes and ECs involved in the growing cancer vascular network and constitute the majority of the recruited cells [76]. Also, to their advantage, CAFs can be activated by BCCs, leading to increased tumor growth [84].

ADSCs in culture with CM from MDA-MB-231 and MCF-7 tumors partly differentiated into myofibroblasts and promoted cancer invasion ability *in vitro* through a TGF-β1/Smad dependent pathway [37]. This depicts bidirectional signaling, reciprocal influence, and consequent phenotype modifications between ADSCs and BCCs [36]. Breast carcinomas often involve a desmoplastic reaction similar to the one found during the healing process in wounds [74]. The EGF/EGFR/Akt-dependent pathway was shown to be involved and the promoting effect reverted after EGF-blockade [98].

Accumulating evidence suggests that chronic inflammation, as found in tumors, is involved in the progression and recurrence of breast cancer [110]. Immune system cells can attract many other host cells, including macrophages and MSCs [111, 112]. Macrophages secrete relevant amounts of MMPs, which increase the invasion ability of cancer [110] and are able to suppress T-cell antitumor effects through a HIF-α dependent pathway [113]. MSCs show inhibitory effects on local immune reaction against breast cancer, with increased T reg ($CD4^+FoxP3^+$) levels in tumors and diminished natural killer cells [90]. Moreover, MSCs are activated to secrete anti-inflammatory cytokines when exposed to proinflammatory cytokines in the tumor milieu, which enables tumor immune evasion [104, 114]. ADSCs isolated from breast cancers also secrete high levels of immunosuppressive cytokines such as IL-4, IL-10, and TGF-β1 [61].

4.4. Cytokines, Chemokines, and Growth Factors: The Influence of Paracrine Signaling versus Cell-to-Cell Contact. To shed light on further mechanisms besides endocrine- and hormone-dependent pathways, several studies have addressed the question of whether cell-to-cell contact promotes breast cancer progression under ADSC influence [23, 46, 58, 88, 104, 115]. ADSCs are known to secrete growth factors, cytokines, and chemokines [15, 93]. Indeed, several factors are increasingly present in the CME, including HGF, IL-6, IL-8, SDF-1, TNF-α, TGF-β1, and VEGF [39, 104]. However, their specific role in breast cancer is still poorly understood, even though some of the mediators such as IL-6 and TGF-β1 seem to be clearly involved in progression of breast malignancies into a more malignant phenotype [39, 106, 116].

In their 2011 published work, Kucerova et al. found that ADSC-CM increased BCC proliferation in a dose-dependent manner, suggesting a cell-to-cell contact-independent mechanism. The CM contained high levels of IL-6, IL-8, MCP-1, and VEGF [93]. Strikingly, coculture of BCCs with CM was more potent in promoting proliferation than direct coculture of the cells. In a recent work, the same authors further investigated the paracrine effects of ADSCs on the triple-negative cell line SKBR-3 and found CM to induce

epithelial-mesenchymal-transition and mammosphere formation, as well as increased cell motility [23]. On the other hand, interestingly, chemosensitivity of BCCs to anticancer drugs was increased by ADSC-secreted factors, which might yield to a potential adjunct for chemotherapeutic protocols. Others found that exosome-mediated cell-to-cell contact was a necessary step for ADSCs to increase tumor cell proliferation [58, 117], with activation of the Wnt pathway as a putative mechanism [46].

4.5. Obesity: Increased ADSC Pool. Obesity is a common condition and has been associated with increased lifetime risk of breast cancer development [118–121]. This has been linked to increased levels of aromatases in WAT and raised levels of estrogen. Surplus adipose tissue worsens the prognosis at onset of breast cancer disease and can contribute to drug resistance [72, 122]. Besides providing energy storage, fat tissue is also regarded as an endocrine organ [123]. In fact, in postmenopausal women, fat remains the most important estrogen production site [124]. WAT is also largely present in the breast and exerts both paracrine and endocrine actions on the mammary gland, as well as any developing BCCs. Leptin, IL-6, TNF-α, IFG, and other hormones are upregulated in obese women and contribute to a state of "chronic inflammation" [44, 125], which can promote breast cancer growth [126]. Indeed, IL-6 has been shown to promote invasion capability and is a marker for poor outcome in breast cancer patients [39, 42, 127–129] and increased IL-6 serum levels are associated with increased metastatic spread [130].

Obesity increases the overall availability and circulating number of ADSCs [44]. Overweight mice have higher yields of ADSCs in the blood stream [22]. ADSCs in obese mice differentiated more frequently into tumor-associated adipocytes and promoted tumor growth [39]. In a different setting, BCCs inhibited adipogenesis of ADSCs, which, in turn, responded with increasing proinflammatory signals, rearranging the ECM [36]. However, it is still unclear whether these findings have any relevance in the clinical setting.

Leptin found in obese patients promotes macrophage differentiation, increasing proinflammatory and proangiogenic factor secretion. In a positive feedback loop, increased proinflammatory cytokines increase the amount of preadipocytes, blocking their maturation to adipocytes, which again raises the amount of inflammatory cytokines and leptin levels [131]. This sort of interplay is believed to be able to predispose a patient to malignancy development [72]. The paracrine mechanism for matrix metalloproteinase (MMP)-2, MMP-9, and Twist1 expression is estrogen- and leptin-dependent [72]. Leptin level also correlated with higher recurrence rates in estrogen- and progesterone-receptor positive (ER^+/PR^+) cancers, underscoring its role in increased invasiveness [72, 132]. In addition, as shown by Rhodes et al., BM-MSCs promoted the growth of breast cancer estrogen-independently [29]. The lack of hormone receptors on basal-like BCCs such as MDA-MB-231 and SKBR-3 advocates for the hormone-independent promoting effect of ADSCs in this type of breast cancer. Interestingly, ADSCs from nonobese people had less influence on BCC proliferation [72].

5. Effects of ADSCs on Migration and Metastatic Spread

The spread of breast cancer to distant locations as well as cancer recurrence worsens prognosis and patient survival drastically and eventually accounts for most breast cancer-related deaths [133]. To metastasize, cancer cells need to go through a process, including invasion, migration through stroma, extravasation, and engraftment in a remote, new niche [134]. This happens directly into adjacent skin and muscle or indirectly through the lymphatic system or blood stream. Frequent distant metastasis sites are bone, brain, lung, and liver [133]. Bone marrow in the skeleton has been attributed to the promotion of growth of breast cancer metastases, due to the presence of a heterogeneous marrow stroma including MSCs, EPCs, hematopoietic stem cells, and fibroblasts among other types of cells, creating a particularly suitable environment for proliferation. Thus, it is essential to investigate and shed light on the effects that fat transplantation to the breast and, more specifically, comprised or implemented ADSCs might have in promoting breast cancer invasion and progression. Several publications report the potential enhancing effect of ADSCs on the metastatic sequence of breast cancer [30, 39, 41, 42, 49, 71].

5.1. ADSCs Influence on Invasion and Migration. A multitude of ADSC-secreted factors are potentially able, alone or in combination, to induce enhanced migration and invasiveness of breast cancer cells. IL-6, IL-8, MCP-1, RANTES, SDF-1, TGF-β1, and VEGF, among others, can shift BCCs to a more aggressive cancer phenotype, resulting eventually in increased metastatic occurrence [5, 93, 135].

SDF-1 is one important factor involved in the spread of BCCs [31, 135]. Blocking CXCR-4 receptors significantly revert the effect, even in the presence of BM-MSCs [31]. The SDF-1 pathway especially is relevant to breast cancer metastasizing to bone [136]. Importantly, CXCR-4 is also linked to poor clinical outcome in patients with breast cancer [135]. In a similar fashion, ADSCs promoted BC spread through a SDF-1-dependent mechanism both *in vitro* and *in vivo* [30]. RANTES is another relevant factor secreted by ADSCs involved in BCC migration [26, 30, 38]. Indeed, Karnoub et al. described previously that BM-MSCs produce RANTES when stimulated by BCCs, which in turn enhances their motility and favors metastasizing [26]. A similar effect could be expected for ADSCs as well. IL-6 and IL-8 are interleukins linked to increased cancer invasion and migration [73, 137, 138]. Additionally, loss of ER has been found to correlate with IL-8 upregulation and breast cancer progression in ER^- breast cancer cell lines [139]. Secretion of MMPs by MSCs fosters breast cancer invasion and migration through ECM modification. MMPs, a class of proteases, are involved in restructuring the tumor stroma and are increasingly expressed in the CME and believed to increase breast cancer invasion [133, 140–142]. MMP-9, for example, increases metastasis without promoting cancer growth [141]. Similarly, MMP-11 enables BCCs to migrate through complex bidirectional signaling with local adipocytes and ADSCs [140].

There is evidence that MMP-mediated BCC migration depends on interplay between the different types of MMPs and does not rely on a single MMP type [74].

5.2. Epithelial-to-Mesenchymal Transition. One important mechanism by which MSCs have been shown to influence cancer cells is turning premalignant or low malignant cells into an invasive and migratory phenotype through epithelial-to-mesenchymal transition (EMT) [143, 144]. EMT, known as a physiological process during development [145], has also been implicated in lung [146], prostate [147, 148], and breast cancer [23, 41, 60, 70]. During EMT, cells are unleashed from their tight junctions, allowing them to escape into the tumor/stroma complex and move through the ECM, increasing their plasticity [149]. Cell propensity for migration is increased, inducing a switch from *in situ* cancer to invasive cancer types; invasion of blood vessels and production of distant metastases are the consequences [150, 151]. MSCs can induce morphological, functional, and molecular changes in epithelial cancer cells, resulting in downregulation of epithelial-specific markers and increased migration, potentially promoting phenotype shifting and migration, generating migration-enabled CSCs, or both [70, 116, 149, 151].

Both BM-MSCs and ADSCs secrete many factors that participate in inducing EMT in breast cancer [71, 131, 152–154], and some ADSC subpopulations might be more prone to inducing EMT than others [41]. Secreted TGF-β1 and IL-6 especially but also IL-8 and MMPs have been long recognized to release cell-to-cell contacts of breast cancer cells and initiate metastasizing behavior [130, 149, 151, 155–157]. Additionally, hormones like leptin and osteopontin can induce EMT [158, 159].

Upon induction, typical EMT genes are upregulated by MSCs such as Slug, Snail1/2, Smad, and Twist1 [160–162]. This translates in a so-called cadherin-switch, which is a hallmark of EMT, where E-cadherin is downregulated, N-cadherin is upregulated [149, 152, 163], and mesenchymal proteins are induced (e.g., Vimentin and Actin). CAFs are also able to increase invasion and migration of luminal and basal type BCCs through the TGF-β1/Smad pathway [164]. Inhibition of the TGF-β1/Smad complex, indeed, has been found to reduce BM-MSC-mediated breast cancer progression through a repression of MSC-to-CAF differentiation [106].

Besides promoting invasion and metastasis, MSC-induced EMT might confer self-renewal activity to BCCs. Indeed, EMT might be at the basis of distant breast cancer metastatic spread, generating $CD44^+$ CSCs, which are mesenchymal-like cells that can easily migrate into the blood stream and extravasate and metastasize [70, 131, 165, 166]. Moreover, while other tumor cells are more or less susceptible to anticancer therapy, CSCs seem more resistant and involved in progression to hormone-receptor negative and chemotherapy-resistant tumor cells [165, 167–170].

6. Clinical Implications

The concept of grafting fat to the breast for aesthetic and reconstructive purposes originated over a century ago but has been again promoted over the last twenty years [4]. While initial concerns about detection of breast cancer during screening have been refuted, the detection of ADSCs as an active component of the autologous processed graft has raised safety concerns. In general, evidence for ADSC application for breast reconstruction after cancer surgery is not voluminous. Nevertheless, several reports, mostly clinical case series, show no evidence of increased cancer occurrence after lipofilling, pointing out the importance of appropriate oncological follow-up [171–173]. In one of the biggest series of lipograft procedures after breast cancer, no increased recurrence was found during a 10-year follow-up [173], whereas higher recurrence rates were detected for *in situ* breast carcinomas after breast conserving therapy followed by autologous fat grafting in another group [174–176]. It is important to note that these reports focus on autologous fat transfer without added stem cells.

However, many reports differ with regard to patient numbers, patient selection criteria, follow-up length, and use of controls [171–173, 177]. Some case reports suggest fat grafting-related cancer recurrence, even though they fail to prove a direct link [178–180].

The first clinical study that assessed stem cell-enriched fat grafting in the postcancer scenario showed promising aesthetic results and no adverse events such as cancer recurrence [181] during a very limited follow-up period of one year. The study was criticized for only including low-risk patients and for being designed without any controls [182].

Petit et al. published a large multicenter study with a median follow-up of 19.2 months involving 513 breast cancer patients. The authors did not find that autologous fat transfer interfered with radio-oncological follow-up, but pointed out the need for further studies with a strict and long-term oncological follow-up period [174, 175]. The same authors found increased local recurrence in a case-control study of a specific subgroup of patients undergoing surgery for *in situ* neoplasias with subsequent autologous lipofilling for breast recontouring [174]. In 2013, they published an expanded study with a larger cohort and a longer follow-up period and confirmed the preliminary results, suggesting an increased cancer risk in this particular patient collective. The results might be due to an exceptionally low control incidence of local events due to selection bias [176], but other authors also agree that oncological safety could be better elucidated [183–185]. These authors did not find an increased rate of recurrence in the other patients studied, and other published case series of breast fat grafting for reconstruction have not shown an increased rate of local recurrence.

A small number of ongoing clinical trials are assessing outcomes after stem cell-enhanced fat transfer to the breast with a focus on aesthetic results (ClinicalTrial.gov; NCT01756092 and NCT01801878) and oncological results. In the GRATESC trial (NCT01035268), an ongoing prospective randomized, multicenter study started in 2010; the authors aim to investigate local and distant cancer recurrence after lipofilling for breast shape and volume improvement after breast conservative surgery, with a planned follow-up period of five years.

7. Discrepancies between Basic Science and Its Clinical Translation

Overall, data regarding the influence of MSC and more in particular ADSCs on breast cancer cells are controversial. A few preclinical reports show decreased breast cancer cell proliferation with high amounts of MSCs [23, 34, 94], even in highly proliferative cell lines such as MDA-MB-231 [49, 95]. On the other hand, a variety of basic science reports demonstrate a fostering effect of MSCs on breast cancer growth, progression, and metastasis [29, 36, 39, 41, 43, 60, 71]. The majority of these reports are raising concerns regarding the use of ADSC for cell-assisted fat grafting for both aesthetic and reconstructive procedures on the breast. These experimental findings are not well substantiated by clinical data thus far: there are a number of case series and one clinical study pointing out at a higher local breast cancer recurrence after lipofilling [175, 176, 178, 186]. Whether basic science is solely unmasking a potential issue, which eventually is not relevant enough to translate to clinical reality or if, indeed, ADSC-enhanced lipofilling procedures bear oncological risk, is an ongoing discussion.

This discrepancy can have several origins and definitely needs to be addressed prior to routine use of this reconstructive strategy. Many variables of the experimental setup can influence the results. As an example, in the mentioned studies, most of the utilized cells grow fast *in vitro* and form large tumors *in vivo*, which might not reflect the actual clinical reality. More likely, dormant, low active cells remain unrecognized in the tumor bed after unsuccessful surgical therapy than highly proliferative ones. Moreover, freshly isolated primary breast cancer cells from tumor excisates or MPEs [5] should be preferred for preclinical studies, along with matched fat tissue and MSCs/ADSCs from the same patient, as different donor biology can affect the MSC functionality and thus the outcome [71]. Primary breast cancer cells have been shown to have lower doubling times [187] and may have different dormancy status which has to be accounted for as well [5]. The timing of MSC addition to the tumors is another important factor. For example, injecting MSCs three weeks after BCC inoculation showed decreased metastasizing in triple-negative breast cancer [95], which is an important finding, as the chosen delayed timing might more appropriately reflect the clinical scenario of *postcancer* breast reconstruction. High amounts of tumor cells as injected in many experimental studies *in vivo* might not depict the clinical reality as well. Indeed, if residual cancer cells remain *in situ* after breast cancer surgery, it is likely that a low number of BCCs would be exposed to a significantly higher number of ADSCs supplemented to fat grafts at the time of reconstruction. Further, distinct MSC origins and species, as well as different culture conditions [50], 2D culture systems which fail to simulate the CME adequately, the BCC-to-MSC ratio, and the route of administration for *in vivo* studies are additional factors which might contribute to controversial results.

On the clinical side, the actual data has to be carefully analyzed. In our opinion, more clinical data is still needed in strong evidence for safety with the use of cell-enhanced fat transplantation. Generally, the available clinical study data suggest safe application of unprocessed autologous fat grafting. The study of Petit et al., with increased recurrent local events after fat grafting in patients with *in situ* cancer of the breast, so far is the only controlled study demonstrating an increased risk of recurrence in a specific cancer subgroup [176]. Larger controlled clinical trials are warranted and these should avoid any selection bias due to sole inclusion of a "favorable" patient population (i.e., mastectomies), which is likely to provide lower recurrence rates than expected after breast conserving surgery [183]. Additionally, large scale registries, such as the American Society of Plastic Surgeons fat grafting to the breast registry, should be broadly implemented.

8. Conclusions

The majority of experimental studies trend to support the propensity of MSCs and ADSCs in promoting growth, progression, and metastatic spread of residual or *de novo* breast cancer after resection. In contrast, only a few clinical case series and trials are reflective of similar findings.

Two scenarios are of interest. (1) Any residual unresected microscopic tumor foci persisting after mastectomy could be activated by ADSCs used in postsurgical restoration. (2) Occult dormant cancer cells in patients with no diagnosed breast cancer but undergoing ADSC therapies for breast augmentation may undergo a malignant transformation.

Currently, the concerns of safety and the debate on efficacy versus such unresolved risk remain ongoing until larger randomized and controlled clinical trials shed light on the scenario. Multiple recommendations based on extensive reviews are available and may be useful for patient information and selection. Overall, most of these studies do not support using autologous stem cell-enhancement at the present [185, 188–190], whereas whole fat grafting appears to be safe in many circumstances.

Abbreviations

ADSC: Adipose-derived stem cell
BCC: Breast cancer cell
BM-MSC: Bone marrow-derived stromal cell
CAF: Cancer-associated myofibroblast
CME: Cancer microenvironment
CSC: Cancer stem cell
CM: Conditioned medium
EC: Endothelial cell
EPC: Endothelial progenitor cell
EMT: Epithelial-to-mesenchymal transition
ER: Estrogen receptor
MMP: Matrix metalloproteinase
MSC: Mesenchymal stem (stromal) cell
MET: Mesenchymal-to-epithelial transition
MPE: Metastatic pleural effusion
PR: Progesterone receptor
SVF: Stromal vascular fraction
WAT: White adipose tissue.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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